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CHES1 (checkpoint suppressor 1) is sufficient to restore the DNA damage-induced G_2 arrest in *S. cerevisiae* checkpoint mutants (Pati *et al*, 1997). Our data suggest that Ches1 functions in an alternative checkpoint pathway. Our goals are to identify genes constituting this pathway, to isolate the human counterparts, and to compare their structure and activity in normal and cancer tissues.

In order to identify the genes involved in the alternative checkpoint pathway, we mutagenized a checkpoint-deficient strain expressing *CHES1*. Mutagenized colonies exhibiting checkpoint phenotypes in the presence of *CHES1* . were further characterized, and represent mutations in genes involved in DNA recombination and repair, gene silencing, microtubule stability, and protein phosphorylation status.

We identified proteins that interact with a functional GST-Ches1 fusion protein. The assay revealed Sin3, a component of the Sin3/Rpd3 histone deacetylase complex. Deleting either *SIN3* or *RPD3* in checkpoint mutants mimics the suppressive effects observed when *CHES1* is expressed in checkpoint mutant strains containing wild-type *SIN3* or *RPD3*. In addition, we screened a human fetal brain library via the two-hybrid approach and identified SKIP, which associates with histone deacetylase complexes in human cells. These data suggests that Ches1 may function similarly in yeast and mammalian cells by acting through a histone deacetylation.

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Introduction

When DNA damage occurs, checkpoints arrest the cell cycle and may result in an array of cellular responses, including activation of DNA repair pathways, increased transcription, alteration of telomere status, and apoptosis (reviewed in Hartwell and Weinert, 1989; Zhou and Elledge, 2000). Multiple genes required for the *Saccharomyces cerevisiae* G_2 checkpoint have been identified, and examination of their mutant phenotypes suggests that a complex signal transduction pathway exists for responses to DNA damage, incomplete DNA replication, or both.

To identify genes required for the human G_2 checkpoint, our laboratory performed a high-copy suppressor screen of a human library to identify cDNAs that suppress *S. cerevisiae* mutant phenotypes resulting from mutations in either *RAD9* or *MEC1* (Pati *et al.*, 1997). Yeast containing *cdc9-8*, the temperature-sensitive allele of DNA ligase, exhibit a maximum permissive growth temperature of 32°C; however, strains containing *cdc9-8* in combination with either the *rad9Δ* or *mec1-1* mutant allele will only grow at 24°C due to loss of the checkpoint function that recognizes and compensates for the partial activity of the *cdc9-8* mutant protein at 32°C. The *cdc9-8, rad9Δ* and *cdc9-8, mec1-1* strains were transformed with a human cDNA library and subsequently screened for growth at 32°C. One cDNA clone, transformant 23 (*tx23*), was shown to confer plasmid-dependent growth at 32°C in both the *cdc9-8, mec1-1* and *cdc9-8, rad9Δ* strains.

Secondary assays revealed that *tx23* is able to suppress multiple checkpoint phenotypes in several checkpoint-deficient strains of the *rad9/mec1* epistasis group. Specifically, *tx23* confers increased survival after exposure to ultraviolet irradiation (UV), ionizing irradiation (IR), and methyl methanesulfonate (MMS). Furthermore, *tx23* can reconstitute the normal DNA damage induced G_2 arrest absent in checkpoint-deficient strains even in a *mec1Δ* mutant strain, which is normally non-viable.

Analysis of *tx23* revealed a 600 bp ORF later named *CHES1* (Checkpoint Suppressor 1), which was shown to encode a truncated portion of a 491 amino acid protein that is a member of the forkhead/winged helix family of transcription factors. Sequence analysis revealed a DNA binding domain common among these transcription factors; however, the DNA binding domain is not present in the original construct identified in the suppressor screen. Only the carboxy-terminal portion of *CHES1* lacking the DNA-binding domain is necessary for checkpoint activity.

In our model, wild-type *S. cerevisiae* strains are resistant to DNA damage because of the presence of both the primary *MEC1*-dependent checkpoint pathway and a less active, alternative *MEC1*-independent pathway. Disrupting the *MEC1*-dependent pathway results in strains that exhibit checkpoint phenotypes, and expression of the Ches1 protein suppresses those phenotypes by activating the alternative pathway in yeast (figure 1).

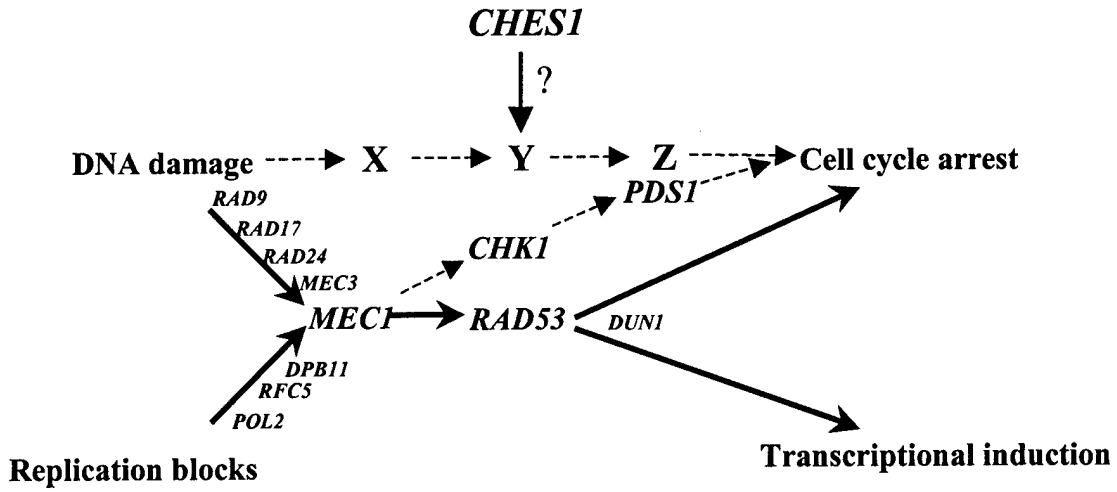

Fig 1. Proposed model for *CHES1* action in cell cycle control and response to DNA damage.

Technical Objective 1

Identify, characterize, and clone the genes in the alternative DNA damage-induced G2 checkpoint of *S. cerevisiae*.

1.A - Perform a comprehensive mutagenesis screen in *S. cerevisiae* to isolate yeast mutant strains that have defective checkpoint bypass genes.

Funding Year 1

The goals for the first year of this grant were to create a temperature-sensitive strain, to optimize mutagenesis conditions, and to perform a mutagenesis screen to identify mutant strains that can no longer be rescued by *CHES1* in the absence of wild type *RAD9*. The method proposed in this project utilizes a temperature-sensitive strain, which contains both the *cdc9-8* and *rad9Δ* mutations. The *rad9Δ* mutation not only lowers the permissive temperature of *cdc9-8* from 32°C to 24°C, but also provides the UV-sensitive phenotype. The *cdc9-8* mutation was gap repaired out from the 9085-1-10-4 strain and put onto an integration vector containing a *URA3* marker. The plasmid was then introduced into a *rad9Δ* strain and plated onto URA deficient- and 5-fluoroorotic acid (5-FOA)-containing media sequentially. This allowed isolation of a strain in which the *cdc9-8* allele replaces the wild type allele of *CDC9* in a *rad9Δ* background (*cdc9-8*, *rad9Δ*).

The amount of ethane methyl sulphonate (EMS) required to yield 50% killing in the *cdc9-8*, *rad9Δ* strain was determined to insure optimal mutagenesis conditions. The mutagenesis screen

was carried out by a slightly modified method than described in the original grant proposal. Specifically, the *cdc9-8, rad9Δ* strain was transformed with two plasmids, each carrying *CHES1* or *RAD9*, before EMS mutagenesis. The transformed strain was mutagenized, followed by washing and plating onto selective media at 30°C. The mutants were replica-plated onto 5-FOA-containing media to select against the *RAD9* plasmid and screened for growth at 24°C but not at 30°C. The colonies identified on this first pass were then patched and replica-plated to retest for temperature-sensitivity. Our model predicts that *chb* (checkpoint bypass) mutants should be not only temperature-sensitive, but also UV-sensitive even when *CHES1* is present in this *cdc9-8, rad9Δ* strain. Therefore, positive clones were subjected to a secondary screen to identify those clones that lost their *CHES1*-dependent response to UV irradiation. The UV sensitivity screen was performed by quantifying the survival rate after exposure to 10 J/m^2 of UV radiation, followed by comparing the isolated *chb* mutants to the parental *cdc9-8, rad9Δ* strain transformed with either empty vector or *CHES1*-containing plasmid.

The screen resulted in 310 putative temperature-sensitive mutants of the approximate 220,000 mutagenized clones, and only three clones exhibited true temperature-sensitive phenotypes after retesting. Among those, *chb13* and *chb16* are highly temperature-sensitive whereas *chb57* is a weaker mutant. With regard to UV-sensitivity, *chb13* and *chb57* are also sensitive to UV radiation, but the effect of *chb16* is intermediate when compared to the controls.

Funding Year 2

During the second year, we attempted to clone the genes mutated in the characterized *chb* mutants isolated from the EMS mutagenesis screen. We confirmed that all three mutants are recessive by mating to wild type strains. In addition, we determined that all three *chb* mutants belong to the same complementation group (i.e., they may contain mutations in the same gene). Therefore, we decided to clone the gene mutated in *chb13,* which exhibits the strongest phenotype. The *chb13* mutant strain was backcrossed twice and selected for mutant phenotype in the presence of *CHES1* to segregate out any unrelated mutations in the genome. The resulting strain was used for complementation analysis by use of a CEN/TRP yeast genomic library. Thirty-four of the 57,500 transformants screened exhibited high-temperature growth; however, most of these clones contained the *CDC9* genomic fragment, which would be expected from this type of analysis. The remaining clones could not complement the mutant phenotype upon transformation back into the *chb13* mutant strain. Similar results were obtained after screening 20,000 and 36,300 transformants of the *chb57* and *chb16* mutant strains, respectively.

Funding Year 3

As described above, we were unable to identify the gene(s) mutated in the *chb* mutant strains by complementation and other analyses of these mutants. The segregation pattern for these mutants is complex and may represent more than one mutation in each strain, which may explain the difficulty in cloning the mutations. Based on these results, we made the decision to modify the mutagenesis protocol by performing insertional mutagenesis with the "Snyder" libraries. The strategy involves transforming the *cdc9-8, rad9Δ* strain containing a *CHES1* expression vector with a library of *S. cerevisiae* genomic clones each containing a *LEU2* marked Tn3 transposon insertion. Individual isolates, which represent homologous recombination events into the genomic locus for each insertion, are selected on media lacking leucine. These transformants

were screened following the same protocol developed for the EMS mutagenesis strategy, and mutant strains resulting from this transformation were further analyzed for checkpoint defects. The disrupted gene can be directly cloned by use of the *LEU2* marker inserted at the site of the mutation; therefore, we will avoid the problems experienced by the EMS methodology.

The mutagenesis screen is approximately 35% complete and has yielded 12 positive clones (Table 1), and the region of disrupted DNA in each mutant was identified by vectorette PCR (Burns *et al.*, 1994). After determining which mutants represent null mutations, we will disrupt the gene of interest in a non-mutagenized strain for further analysis. In several of the isolated mutants, the insertion disrupted the region either 3- or 5-prime of one or two genes. In those cases, we will first determine if the insertion results in an increased or decreased expression for each gene before proceeding with further analysis. These clones may also represent mutants that contain multiple insertion sites; therefore, we will perform Southern analysis using the transposon region as a probe to identify those clones that underwent multiple insertion events.

Mutant	**Disrupted ORF**	**Position of Insertion**	**Brief Description of Function**
cbm24	*MEF1*	Central	Mitochondrial translation-initiation factor.
cbm154	*SUM1*	Central	Involved in chromatin silencing; represses genes necessary for meiosis during mitosis; levels regulated by the pachytene checkpoint.
cbm128	*YOL045W*	C-terminal - disrupts kinase domain	Serine/threonine protein kinase of unknown function.
cbm77	*MDM20*	N-terminal	Controls assembly/stabilization of actin cables; involved in cytoskeleton organization, mitochondrial inheritance, and mitotic spindle assembly/maintenance.
cbm42	*ENA1* & *RSM10*	Region 5-prime of ATG start	*Ena1*: (Crick strand) - P-type ATPase involved in Na^+ and Li^+ efflux. *Rsm10*: (Watson strand) - Component of the mitochondrial ribosomal small subunit.
cbm166	*GYP6* & *YJL045W*	Region 3-prime of stop	*GYP6*: (Crick strand) – Rab-like GTPase-activating protein (GAP) for Ypt6, which is involved in protein transport. *YJL045W* (Watson strand)- Probable succinate dehydrogenase.
cbm130	*IES3* & *YLR053C*	Region 3-prime of stop	*IES3* (Watson strand) – unknown function. *YLR053C* (Crick strand) –unknown function.
cbm135	*GAD1*	N-terminal	Glutamate decarboxylase; Required for normal oxidative stress tolerance.
cbm74	*TAP42*	C-terminal	Essential protein that binds type 2A and

			type2A-related phosphatases.
cbm112	*CIN5*	Central	Basic-leucine zipper protein; transcriptional activator required for microtubule stability; required for protection against DNA alkylating agents; may be involved in silencing.
cbm99	*SRS2*	Central - disrupts helicase domain	ATP-dependent DNA helicase (3'to 5') involved in DNA repair and recombination; Phosphorylated in response to damage in a checkpoint-dependent manner; required for proper timing of commitment to meiotic recombination and meiosis.
cbm289	*CDC14*	Region 5-prime of ATG start	Tyrosine-specific protein phosphatase; required for mitosis and sporulation.

Table 1. Mutant strains isolated from the insertional mutagenesis screen to identify genes necessary for the *CHES1*-dependent checkpoint.

1.B - Candidate gene approach: Investigating genes that may potentially function in the alternative checkpoint pathway.

Funding year 2

We took the candidate gene approach by examining genes that may potentially function in the alternative checkpoint pathway. The *S. pombe* and mammalian homologs of the *S. cerevisiae* Chk1 protein function in response to DNA damage and are necessary for checkpoint function; therefore, we disrupted *CHK1* in a strain of the same genetic background as in the *chb* mutants. Disruption of *CHK1* did not result in the *chb* phenotype, and over-expression of wild type *CHK1* and *CHES1* in *chb* mutants did not complement the mutant phenotype.

Another candidate gene, *TEL1*, is involved in regulating telomere maintenance and is homologous to the *S. cerevisiae* and mammalian checkpoint genes *MEC1* and *ATM*, respectively. Southern blot analysis showed that expression of *CHES1* has no affect on telomere length in either wild type or *tel1Δ* strains. Furthermore, over-expression of wild type *TEL1* and *CHES1* in *chb* mutant strains did not complement the mutant phenotype. Overexpression of *CHES1* suppressed both the temperature- and UV-sensitivity exhibited by the *cdc9-8*, *rad9Δ*, *tel1Δ* mutant, therefore the alternative pathway is still intact and must not require Tel1. These data suggest that neither *CHK1* nor *TEL1* are part of the alternative DNA checkpoint pathway.

Funding year 3

We continued the candidate gene approach by creating a null allele of *TOF1,* which interacts with topoisomerase I and is implicated to take part in a *RAD9*-independent genotoxic stress response pathway that functions specifically in S-phase (Foss, 2001). We were unable to assay

CHES1's affect on the checkpoint phenotypes in the *cdc9-8*, *rad9*Δ, *tof1Δ* triple mutant, because surprisingly, deletion of *TOF1* suppressed the temperature-sensitive phenotype in the absence of *CHES1* (data not shown). The *cdc9-8*, *rad9*Δ, *tof1Δ* mutant grows similar to wild type at 32°C and is viable up to 35°C. Overexpression of *TOF1* in the *cdc9-8*, *rad9*Δ, *tof1Δ* mutant reverses the effect thereby decreasing the permissive temperature back to 32°C. To date, nothing further has been done to explore this interesting observation.

Funding year 4 (no cost extension)

Knowing the subcellular localization of Ches1 in *S. cerevisiae* would provide insight on how and with which proteins Ches1 might function, thus narrowing the range of candidate Ches1-interacting genes. Furthermore, determining the localization of Ches1 might help to shed light on why full-length *CHES1* and yeast two-hybrid (Y2H) DNA-binding bait constructs fused to the carboxy-terminus of *CHES1* are toxic when expressed in yeast. We created a vector containing a GFP-Ches1 (c-terminal region) fusion insert to monitor its subcellular localization. Results indicate that the functional GFP-Ches1 fusion protein localizes to the nucleus (Figure 2). Therefore, functional Ches1 localizes to the nucleus, and the DNA-binding activity exhibited by full-length Ches1 and the Y2H constructs are most likely the cause of toxicity when expressed in *S. cerevisiae*. Furthermore, PredictNLS server (http://dod0.bioc.columbia.edu/cubic/predictNLS) predicts a high-scoring NLS in the c-terminus of Ches1, which is found in 193 proteins, 98% of which are localized to the nucleus.

1.C - Perform biochemical assays to identify yeast and human proteins that interact either directly or indirectly with Ches1.

Funding year 3 and funding year 4 (no cost extension)

1.C.1 - Yeast Two-Hybrid Screens:

We moved to identifying proteins that directly interact with the Ches1 protein using biochemical methods. After creating the appropriate *CHES1* bait vector and confirming expression by Western analysis, we screened both an *S. cerevisiae* and a human fetal brain cDNA library via a cytoplasmic yeast two-hybrid system (Stratagene).

***S. cerevisiae* library results**: We screened approximately 2,250,000 clones and isolated 115 prey-dependent positive clones. All clones were determined to be bait-independent and therefore represent false-positive interactions. Stratagene recommends that those using the cytoplasmic yeast two-hybrid system not screen *S. cerevisiae* cDNA libraries, as the nature of the system promotes the isolation of many false-positives.

Human library results: We screened approximately 1,000,000 clones and identified one bait-dependent positive clone. The prey cDNA encodes *SKIP* (Ski-interacting protein), which regulates transcription through interactions with HDAC, CBF1, mSin3A, SMRT, and

the SMAD proteins (Zhang *et al.*, 2001; Leong *et al.*, 2001; Zhou (a) *et al.*, 2000; Zhou (b) *et al.*, 2000).

1.C.2 - Glutathione-S-Transferase (GST) Pulldown Assays:

In addition to the two-hybrid screens, we have constructed a yeast expression vector encoding a GST-*CHES1* fusion protein as a means of identifying Ches1-interacting proteins. Western blot analysis using anti-GST antibody confirmed expression of the expected 55kD GST-*CHES1* fusion protein. Furthermore, the fusion protein retains the ability to suppress the checkpoint defects observed in the *cdc9-8, rad9Δ* strain thereby allowing growth at 32°C and increased survival after UV irradiation (data not shown). Protein extracts from a protease-deficient yeast strain expressing either the GST-*CHES1* fusion protein or GST alone were used for GST pulldown assays using Sepharose-glutathione beads. We successfully enriched for the fusion protein and identified three potential Ches1-interacting proteins, which were sequenced by mass spectrometry (Figure 3):

- Sse2 – Heat shock protein of the HSP70 chaperone family.
- Ssb2 – Heat shock protein of the HSP70 chaperone family.
- Sin3 – Paired amphipathic helix protein that complexes with Rpd3 to form the Rpd3/Sin3 locus-specific histone deacetylase complex. Dimerization of Sin3 with Rpd3 is essential for deacetylase activity. Sin3 and Rpd3 are well conserved in humans (human mSin3A and HDAC1/HDAC2, respectively).

  The finding that Ches1 interacts with the Sin3/Rpd3 histone deacetylation complex may help to explain why full-length *CHES1* and yeast two-hybrid (Y2H) DNA-binding bait constructs fused to the carboxy-terminus of *CHES1* are toxic when expressed in yeast. It is possible that if Ches1 does indeed interact with Rpd3/Sin3 in yeast, then the toxicity may result from Ches1 promoting the regional silencing of essential loci located in the proximity of the element to which the DNA-binding domain binds. Conversion of a locus-specific repressor to a regional silencer by a single amino acid substitution has been reported (Rusche and Rine, 2001).

Funding year 4 (no cost extension)

We have disrupted both *SIN3* and *RPD3* in a *cdc9-8, rad9Δ* background to assess whether Sin3/Rpd3 deacetylase activity is required for the *CHES1*-mediated checkpoint response. Results indicate that both the *cdc9-8, rad9Δ, sin3Δ* and *cdc9-8, rad9Δ, rpd3Δ* triple mutant grow at the non-permissive temperature of 32°C even in the absence of *CHES1* expression (Figure 4). Furthermore, deleting *SIN3* and *RPD3* in a *rad9Δ* background suppressed the UV-sensitivity phenotype normally observed in *rad9Δ* mutants (Figure 5), and this suppression is similar to the suppressive effects observed in a *rad9Δ, SIN3, RPD3* strain expressing *CHES1* (Pati *et al.*, 1997). Therefore, these data suggest that Ches1 may play an inhibitory role by

interacting with Sin3, which results in an altered Sin3/Rpd3 deacetylase activity leading to checkpoint activation.

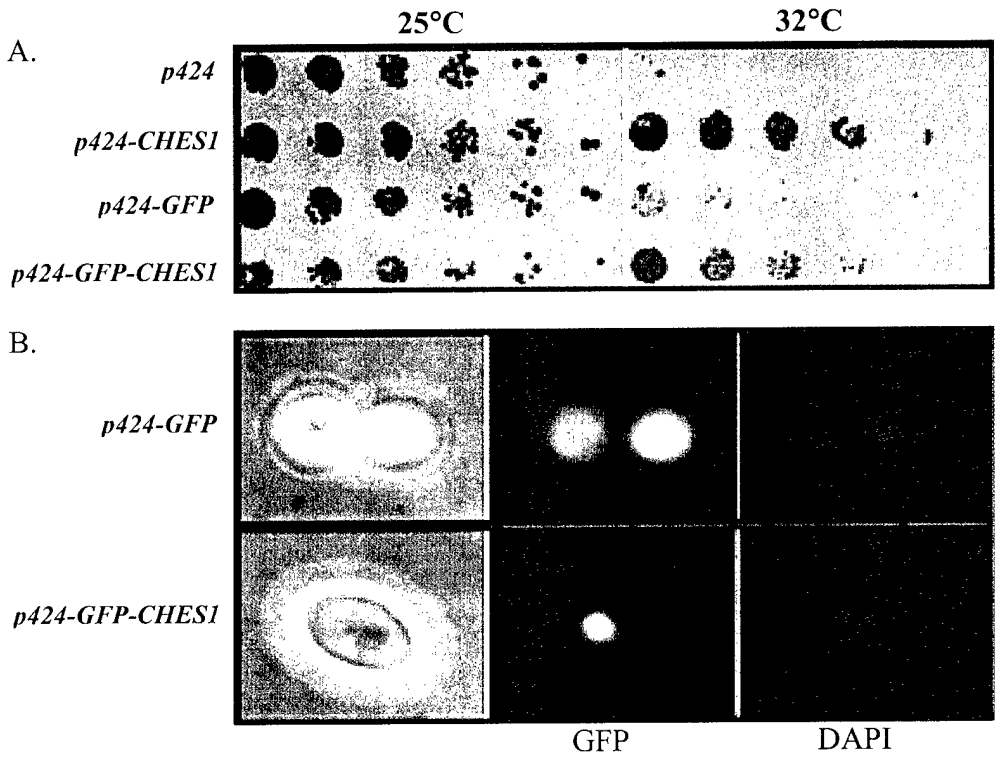

Fig 2. Subcellular localization of Ches1. (A) The carboxy-terminal region of *CHES1* rescues the temperature-sensitive lethality of a *cdc9-8, rad9*Δ mutant strain for growth at 32°C when expressed alone (*p424-CHES1*) or as a GFP fusion protein (*p424-GFP-CHES1*), while the respective empty vectors do not (*p424* and *p424-GFP*). (B) The GFP-Ches1 fusion protein (*p424-GFP-CHES1*) localizes to the nucleus when expressed in *S. cerevisiae*, while GFP alone (*p424-GFP*) localizes to the cytoplasm.

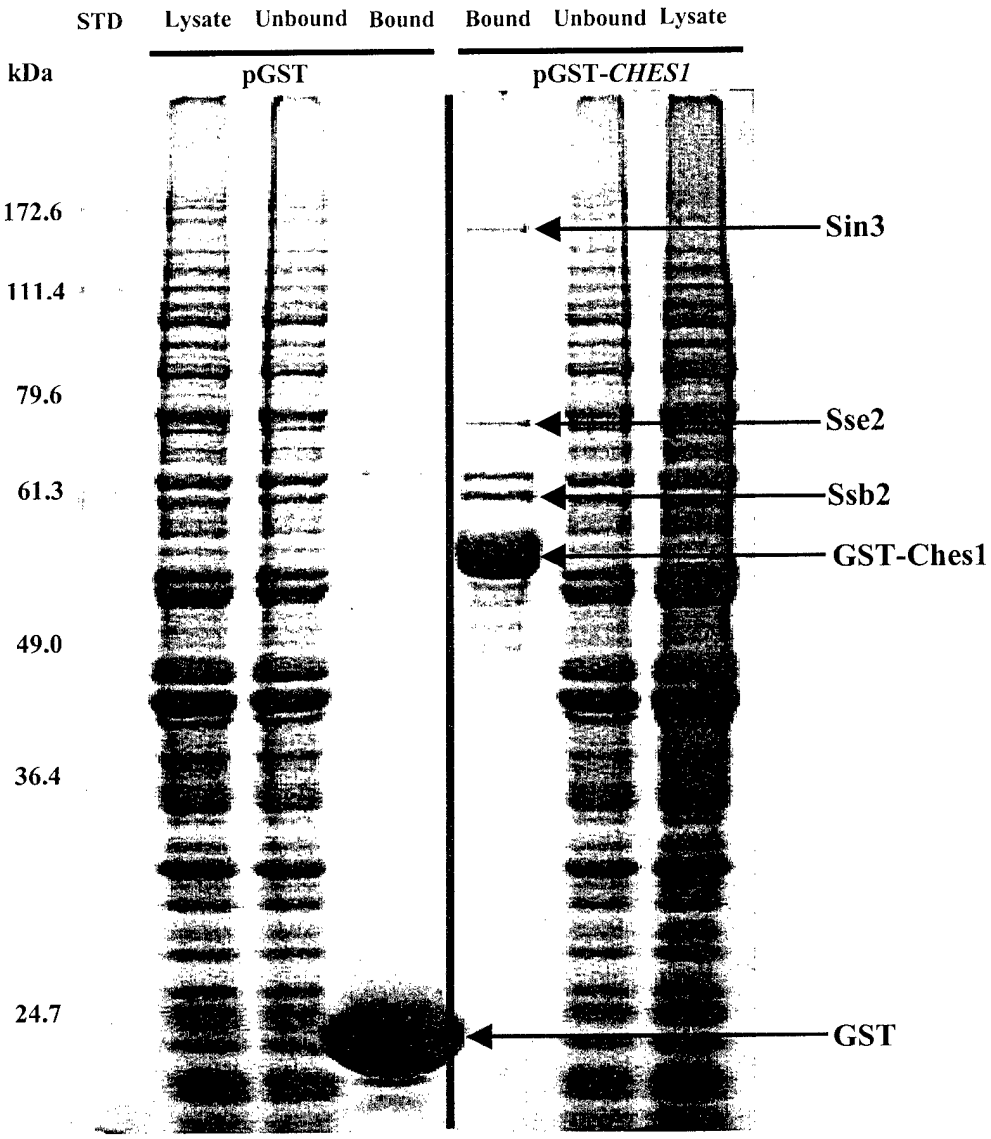

Fig 3. Glutathione-S-Transferase (GST) pulldown assay to identify Ches1-interacting proteins. Protein lysates were prepared from yeast expressing GST alone (pGST) or GST-*CHES1* (pGST-*CHES1*) and incubated with Sepharose-glutathione beads. The beads were washed, collected, boiled, and separated by SDS-PAGE for Coomassie staining.

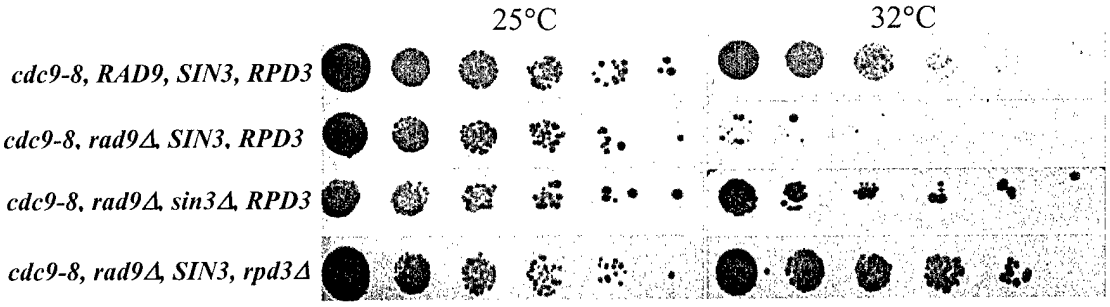

Fig 4. Deletion of *SIN3* or *RPD3* suppresses a *cdc9-8, rad9Δ* mutant. Each strain was grown to log phase and spotted at 5-fold dilutions onto YPD and incubated at either 25°C or 32°C.

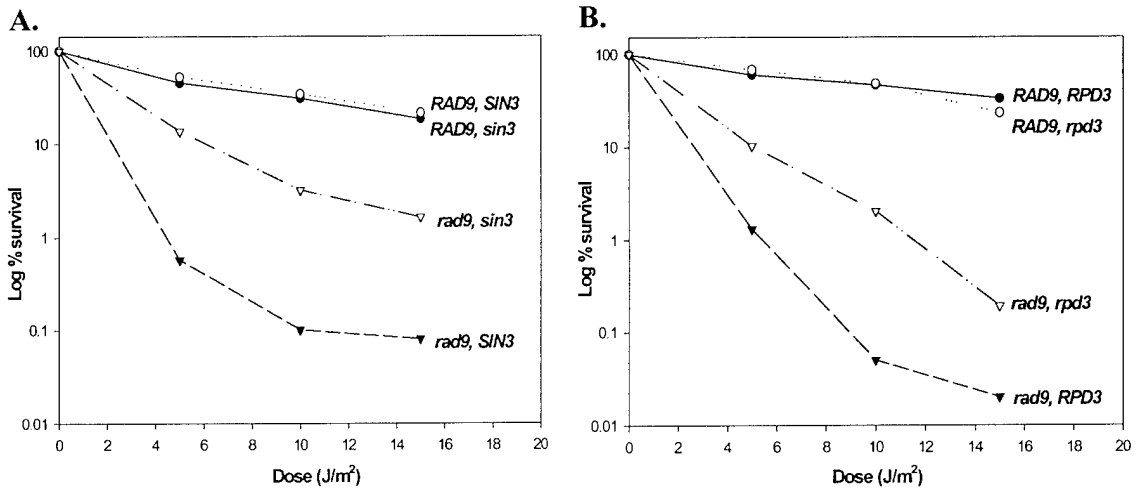

Fig 5. Deletion of *SIN3* or *RPD3* suppresses the UV-sensitivity exhibited by a *rad9Δ* checkpoint deficient strain. Strains containing wild-type or mutant alleles of (A) *RAD9* and *SIN3* or (B) *RAD9* and *RPD3* were grown to log phase, plated, and UV-irradiated.

Expression of *CHES1* is sufficient to suppress *mec1Δ* lethality, and the *CHES1*-mediated checkpoint is functional in a *mec1Δ* mutant strain (Pati *et al.*, 1997). If Ches1 functions to inhibit Sin3/Rpd3 activity, then deleting *SIN3* or *RPD3* should mimic Ches1 activity in the absence of *CHES1* expression. To this end, we created *mec1Δ, sin3Δ* + *pMEC1-URA* and a *mec1Δ, rpd3Δ* + *pMEC1-URA* double mutants. Plating these strains onto 0.1% 5-FOA, which selects against the presence of the *pMEC1-URA* covering vector, resulted in viable colonies. Therefore, deletion of either *SIN3* or *RPD3* is sufficient to suppress *mec1Δ* lethality. *mec1Δ, sin3Δ* and *mec1Δ, rpd3Δ* colonies were taken from 5-FOA plates (i.e. no longer contain *pMEC1*) and streaked onto a YPD plate along with the *mec1Δ* + *pMEC1-URA* strain. Replica plating to a plate containing 0.1% 5-FOA and a plate lacking uracil demonstrates that the *mec1Δ, sin3Δ* and *mec1Δ, rpd3Δ* strains are viable and that they no longer contain the *pMEC1-URA* covering plasmid, respectively (Figure 6). In order to assay effect on UV-sensitivity in *mec1Δ* strains lacking *SIN3* and *RPD3*, a high-copy *RNR1* expression plasmid, which

suppresses *mec1Δ* lethality (Zhao *et al.*, 1998), was transformed into the following strains: *MEC1, SIN3, RPD3* (wild-type); *MEC1, sin3Δ; MEC1, rpd3Δ; mec1Δ, SIN3, RPD3; mec1Δ, sin3Δ;* and *mec1Δ rpd3Δ*. Deleting *SIN3* and *RPD3* in a *mec1Δ* background suppressed the UV-sensitivity phenotype normally observed in *mec1Δ* mutants (Figure 7), and this suppression is similar to the suppressive effects observed in a *mec1-1, SIN3, RPD3* strain expressing *CHES1* (Pati *et al.*, 1997). These data further support the idea that Ches1 functionally inhibits Sin3/Rpd3 activity, as deletion of either gene mimics the Ches1-dependent suppression of mec1Δ lethality and UV-sensitivity. Current work is focused on assessing whether checkpoint activity is also restored in the *mec1Δ, sin3Δ* and *mec1Δ, rpd3Δ* mutant strains.

A manuscript is in preparation describing the data resulting from the GST pulldown assay and the subsequent experiments performed to assess the potential role of the Sin3/Rpd3 complex in checkpoint control.

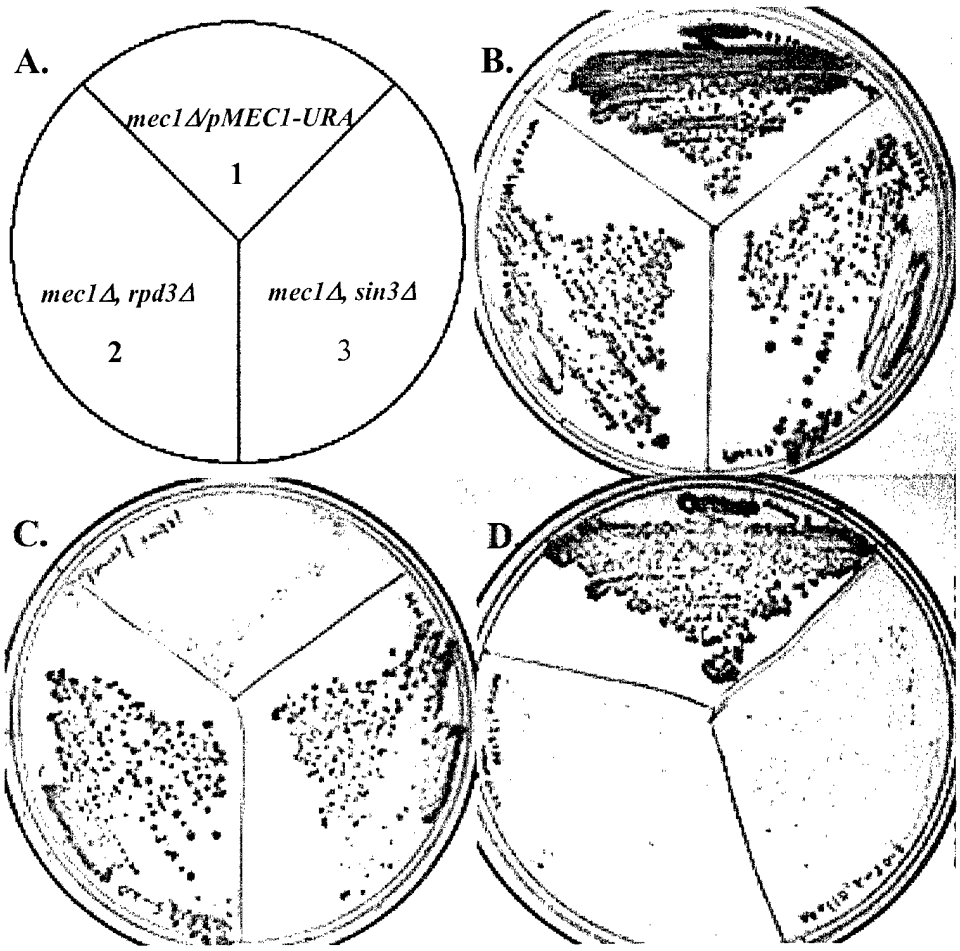

Fig 6. Deletion of *SIN3* or *RPD3* suppresses a *mec1Δ* mutant. (A-1) *mec1Δ* mutant carrying wild-type *MEC1* on a URA vector (*pMEC1-URA*); (A-2) *mec1Δ, rpd3Δ* mutant taken from a plate containing 5-FOA; (A-3) *mec1Δ, sin3Δ* mutant taken from a plate containing 5-FOA. Each strain was replica plated from (B) YPD to (C) a plate containing 0.1% 5-FOA and (D) SC-URA and incubated at 30°C.

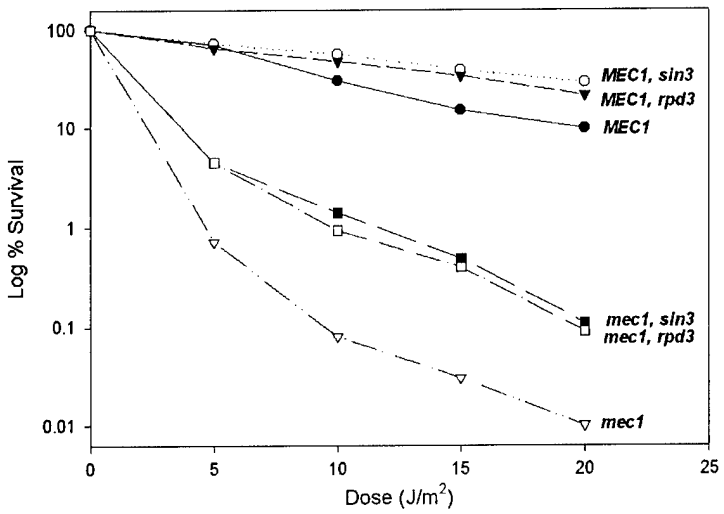

Fig 7. Deletion of *SIN3* or *RPD3* suppresses the UV-sensitivity exhibited by a *mec1Δ* checkpoint deficient strain. Strains containing wild-type or mutant alleles of *MEC1*, *SIN3*, and *RPD3* were transformed with a high-copy *RNR1* expression vector, grown to log phase, plated, and UV-irradiated.

1.D - Assessment of the effect of *CHES1* expression on the *S. cerevisiae* transcriptome by microarray analysis.

Funding Year 4

We have demonstrated that Ches1 both physically and genetically interacts with the *S. cerevisiae* Sin3/Rpd3 deacetylation complex, which controls the transcription of genes throughout the yeast genome. Ches1 may alter the activity of this complex and therefore affect gene expression upon DNA damage; therefore, we decided to perform microarray analysis to identify those genes that are differentially regulated in a Ches1-dependent manner. To this end, we prepared total RNA from the following cultures for hybridization to Yeast Genome S98 Arrays (Affymetrix):

- *cdc9-8, rad9Δ + empty TRP vector* - cultured at 24°C
- *cdc9-8, rad9Δ + p424-TX23-TRP* - cultured at 24°C
- *cdc9-8, rad9Δ + p424-TX23-TRP* - cultured at 24°C + 1 hr shift to 30°C
- *cdc9-8, RAD9 + empty TRP vector* - cultured at 24°C + 1 hr shift to 30°C
- *cdc9-8, RAD9 + p424-TX23-TRP* - cultured at 24°C + 1 hr shift to 30°C

The data obtained from the microarray experiments is currently being analyzed to assess Ches1's affect on transcription. Furthermore, the resulting gene expression profiles will be compared to those resulting from deletion of *SIN3* and *RPD3* as published in Bernstein *et al.*, 2000. If Ches1 inhibits Sin3/Rpd3 HDAC activity, then the Ches1-dependent expression profile should resemble the profile displayed by *sin3Δ* and *rpd3Δ* mutants.

Technical Objective 2

Isolate mammalian homologs of the alternative checkpoint pathway and analyze the structure and expression of the cloned checkpoint genes in human breast cancer derived cell lines and human breast tumor samples.

In the second objective, we proposed to isolate mammalian homologs of the alternative checkpoint pathway and to analyze the structure and expression of the cloned checkpoint genes in human breast cancer derived cell lines and human breast tumor samples. Unfortunately, we were unable to accomplish these goals, as the experiments proposed in the second objective are dependent on data resulting from the first objective. However, recent data described in section 1.C suggests that Ches1 may interact with Skip to control gene transcription.

We have created FLAG-*SKIP* and MYC-*CHES1* (full-length and c-terminal portion) mammalian constructs in order to perform immunoprecipitation assays to confirm the Ches1-Skip interaction. We also plan to perform Western analysis on these protein extracts using commercially available antibodies against HDAC, mSin3A, and possibly other proteins known to associate with the HDAC/mSin3A complex. Future work will be focused analyzing the potential relationship between *CHES1*, histone modification, and checkpoint control in mammalian cells.

Key research accomplishments and reportable outcomes

- Construction of a *cdc9-8*, *rad9Δ* mutant strain, which is both UV-resistant and grows at 32°C in the presence of *CHES1*.
- Isolation of three *chb* mutants that display varying degrees of temperature-sensitivity and UV-sensitivity.
- Exclusion of *S. cerevisiae CHK1* and *TEL1* as parts of the alternative DNA checkpoint pathway.
- The carboxy-terminus of Ches1 localizes to the nucleus in *S. cerevisiae* and is sufficient to restore checkpoint function in checkpoint-deficient strains.
- Implementation of an alternative mutagenesis strategy, specifically by using insertional libraries to aid in identifying those genes mutated in *chb* mutant strains.
- Identification of 12 mutants that may contain mutant alleles of genes required for the *CHES1*-mediated checkpoint.
- Construction of a functional hSOS-*CHES1* fusion vector for use in a cytoplasmic yeast-two-hybrid screen in which human Skip was identified as a possible Ches1-interacting protein.
- Construction of a functional GST-*CHES1* fusion vector for use in GST pulldown assays in which Sse2, Ssb2, and Sin3 were identified as potential Ches1-interacting proteins.
- Construction of *cdc9-8*, *rad9Δ*, *sin3Δ* and *cdc9-8*, *rad9Δ*, *rpd3Δ* mutant strains, which both mimic a *cdc9-8*, *rad9Δ* + *pCHES1* when assayed for UV-sensitivity and temperature-sensitivity.
- Construction of *mec1Δ*, *sin3Δ* and *mec1Δ*, *rpd3Δ* mutant strains. Deletion of *SIN3* and *RPD3* in the *mec1Δ* background suppresses *mec1Δ* lethality.
- Presentations:
    - Plon, S. E., Pati, D., and Li, Y.-C. (1997). Alternative DNA damage checkpoint pathways in eukaryotes. Poster presentation. Ataxia Telangiectasia and ATM: Functional, Genetic and Clinical Ramifications. Baltimore, Maryland.
    - Li, Y.-C. and. Plon, S. E. (1999). Isolation of mutants that are defective in an alternative DNA damage checkpoint pathway. Poster presentation. American Society for Microbiology Conference: Yeast Genetics and Human Disease II. Vancouver, British Columbia, Canada

- Scott, K.L. and Plon, S.E. (2001). Alternative DNA damage checkpoint pathways in eukaryotes. Poster presentation. Graduate School Symposium; Baylor College of Medicine, Houston, Texas.

- Scott, K.L. and Plon, S.E. (2001). Alternative DNA damage checkpoint pathways in eukaryotes. Poster presentation. Department of Molecular and Human Genetics Retreat; Baylor College of Medicine, Houston, Texas.

Conclusions:

Pulldown assays using a *GST-CHES1* expression construct revealed that Ches1 might interact with Sin3, a component of the *S. cerevisiae* Sin3/Rpd3 histone deacetylase complex. The data presented within this report demonstrate that deletion of *SIN3* or *RPD3* mimics Ches1's activity in *S. cerevisiae*, as checkpoint-deficient stains containing *sin3Δ* or *rpd3Δ* alleles display increased survival after damage when compared to the checkpoint-deficient containing wild-type *SIN3* and *RPD3* alleles. Therefore, Ches1's activity in yeast is at least partially dependent on the Ches1-dependent inhibition of Sin3/Rpd3 histone deacetylase activity. In addition, we identified SKIP as a potential Ches1-interacting protein. SKIP is known to associate with members of human HDAC complexes; therefore, Ches1 may function similarly in human cells. A possible model to explain Ches1's function is that Ches1 inhibits Sin3/Rpd3 activity, which results in an altered expression of gene(s) that result in a restoration of the DNA damage-induced G_2 checkpoint absent in checkpoint-deficient mutants. Alternatively, altering deacetylase activity may alleviate chromatin condensation and thus allow DNA damage machinery better access to sites of damaged DNA. We are testing both of these models to help elucidate the role of histone deacetylation in the response to DNA damage.

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Reconstitution of a *MEC1*-Independent Checkpoint in Yeast by Expression of a Novel Human *fork head* cDNA

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A novel human cDNA, *CHES1* (checkpoint suppressor 1), has been isolated by suppression of the *mec1-1* checkpoint mutation in *Saccharomyces cerevisiae*. *CHES1* suppresses a number of DNA damage-activated checkpoint mutations in *S. cerevisiae*, including *mec1*, *rad9*, *rad24*, *dun1*, and *rad53*. *CHES1* suppression of sensitivity to DNA damage is specific for checkpoint-defective strains, in contrast to DNA repair-defective strains. Presence of *CHES1* but not a control vector resulted in G_2 delay after UV irradiation in checkpoint-defective strains, with kinetics, nuclear morphology, and cycloheximide resistance similar to those of a wild-type strain. *CHES1* can also suppress the lethality, UV sensitivity, and G_2 checkpoint defect of a *mec1* null mutation. In contrast to this activity, *CHES1* had no measurable effect on the replication checkpoint as assayed by hydroxyurea sensitivity of a *mec1* strain. Sequence analysis demonstrates that *CHES1* is a novel member of the *fork head*/Winged Helix family of transcription factors. Suppression of the checkpoint-defective phenotype requires a 200-amino-acid domain in the carboxy terminus of the protein which is distinct from the DNA binding site. Analysis of *CHES1* activity is most consistent with activation of an alternative *MEC1*-independent checkpoint pathway in budding yeast.

Eukaryotes have a complex yet conserved response to DNA damage including changes in cell cycle kinetics (23) and transcriptional induction of multiple genes (17). Some of the genes responsible for these DNA damage-inducible cell cycle arrests or checkpoints have been identified (11). In at least one instance, the human checkpoint gene mutated in the disease ataxia telangiectasia, *ATM* (47), has sequence and functional homologs in many eukaryotes, including *Drosophila melanogaster* (*MEI41* [22]), *Saccharomyces cerevisiae* (*TEL1* and *MEC1*/*ESR1* [20, 29, 38]), and *Schizosaccharomyces pombe* (*rad3*, [50]). In contrast, other genes such as mammalian *p53*, a regulator of the damage-inducible G_1 checkpoint (31), do not appear to be conserved in lower eukaryotes. Germ line mutations in human checkpoint genes *ATM* and *p53* result in a predisposition to malignancy, perhaps due to the unstable nature of the genome which results when checkpoint function is absent (reviewed by Weinert and Lydall [61]).

Multiple checkpoint genes in both budding yeast (*RAD17*, *RAD24*, *RAD9*, *RAD53*/*MEC2*/*SAD1*, *MEC3*, and *POL2* [4, 23, 60, 62]) and fission yeast (*hus1*, *hus3*, *rad1*, *rad9*, *rad17*, *rad24*, *rad25*, *rad26*, *cds1*, and *chk1* [2, 3, 13, 16, 28, 37, 45, 52]) are required for normal checkpoint function. Examination of these mutant phenotypes reveals that certain gene products, e.g., Mec1 (60) and Rad3 (28), are required for response to both incomplete replication and DNA damage. Other mutant strains have specific defects in the response to DNA damage, e.g., *rad9*, *rad24* (60) of *S. cerevisiae* and *chk1* (56) and *rad27* (3) of *S. pombe*. The number of proteins required for establishment of cell cycle arrest after DNA damage suggest that a complex signal transduction pathway exists from the initial detection of DNA damage to cell cycle arrest and transcriptional induction in eukaryotic cells (7, 30).

Although substantial information has been obtained on the control of the G_1 checkpoint in mammalian cells, human homologs of many of the genes responsible for the G_2 checkpoint in yeast have not yet been identified (reviewed by Hawley and Friend [24]). In particular, few of the gene products required for the G_2 arrest in mammalian cells after DNA damage have been elucidated. The human homolog of the *S. pombe rad9* checkpoint gene has been recently isolated by sequence homology, and expression of the cDNA results in partial suppression of a *rad9* mutant (34). We have developed a screen for the isolation of new human checkpoint cDNAs by demanding high-copy suppression of several checkpoint mutations in *S. cerevisiae*. In this way, human cDNA libraries can be effectively screened to obtain both homologous cDNAs and cDNAs which may function further downstream in the checkpoint pathway. This screen has resulted in the isolation of a novel member of the *fork head*/Winged Helix family of transcription factors. Expression of this clone results in reconstitution of the G_2 checkpoint in a *mec1*-independent manner.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used for these experiments are described in Table 1. The source of each strain other than this laboratory is indicated.

Yeast transformation. Logarithmic cultures of the indicated strain were transformed by a modification of the method of Schiestl and Gietz (48) in which the DNA and 50% polyethylene glycol solution are added directly to the yeast in lithium acetate without any preincubation. Plasmid DNA from yeast was extracted by glass bead disruption (25) and transformed into *Escherichia coli* by electroporation (Bio-Rad, Hercules, Calif.).

Libraries and DNAs. The cDNA library and ADANS vector for screening in yeast were obtained from John Colicelli (8, 9). Adrenal and lymphocyte human libraries used to obtain longer clones were constructed by S. E. Plon and Joshua La Baer (MGH Cancer Center), respectively. Screening of the library in yeast was performed as described previously (42) by transformation of the recipient strain with purified library DNA. The transformed cultures were divided onto leucine-deficient plates and left at 23°C for 14 to 18 h, after which the plates were transferred to 30°C. Colonies were isolated after 4 to 6 days of growth. DNA was isolated as described above and retransformed into the recipient strain to determine plasmid dependence for suppression of the checkpoint phenotype. Sequencing of both strands of the *CHES1* cDNAs was performed both manually and on a Licor automated sequencer after subcloning into pBluescriptKS+.

Survival determination after methyl methanesulfonate (MMS), hydroxyurea (HU), UV light, and X-ray exposure. For all treatments, logarithmically growing

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TABLE 1. Yeast strains used in these experiments

Strain	Genotype	Source other than our lab
171-10-2	*MAT***a** *cdc9-8 mec1-1 leu2 ura3 ade2 ade3 trp1*	T. Weinert
121-1-1b	*MAT*α *mec1-1 leu2 his3 his7 ura3*	T. Weinert
9085-1-7-3	*MAT***a** *leu2 his3 ura3*	
9085-1-8-3	*MAT*α *cdc9-8 leu2 ura3 trp1*	
9085-1-10-4	*MAT*α *cdc9-8 leu2 his3*	
9085-1-2-3	*MAT***a** *rad9::HIS3 leu2 ade2*	
10011-2-1	*MAT***a** *rad6 leu2 ura3*	
CRY1	*MAT***a** *can1-100 ade2 his3 leu2 trp1 ura3*	S. Elledge
CRY2	*MAT*α *can1-100 ade2 his3 leu2 trp1 ura3*	S. Elledge
Y286	*MAT*α *dun1-Δ100::HIS3 can1-100 ade2 his3 leu2 trp1 ura3*	S. Elledge
Y301	*MAT***a** *rad53-21(sad1-1) can1-100 ade2 his3 leu2 trp1 ura3*	S. Elledge
Y438	*MAT***a** *rad9::HIS3 can1-100 ade2 his3 leu2 trp1 ura3*	S. Elledge
Y669	*MAT***a** *mec1::HIS3 can1-100 ade2 his3 leu2 trp1 ura3* + PWJ81 (containing the *MEC1* gene and *URA3* marker)	S. Elledge
EMY62	*MAT***a** *rad52::URA3 his3 leu2 trp1 ura3*	D. Botstein
XKS255-1A	*MAT*α *rad24-1 his3 leu2 lys2 trp1 ura3*	J. Game

cultures were obtained by inoculation of 10 to 20 ml of leucine-deficient medium with an aliquot of a stationary-phase culture and grown overnight. The next morning, cultures containing between 1 million and 5 million cells/ml were used for the experiments.

(i) MMS. Cultures were incubated at indicated times in the presence and absence of media containing 0.1% MMS (Sigma, St. Louis, Mo.) at 30°C. Following the incubation, cells were briefly sonicated and plated onto leucine-deficient plates, colonies were counted in 48 to 72 h, and percent survival was determined.

(ii) UV irradiation. Cells were diluted in leucine-deficient medium at different concentrations, briefly sonicated, and then plated. Plates were irradiated at indicated doses, using a Spectronics (Westbury, N.Y.) Spectrolinker UV crosslinker. Plates were incubated at 30°C for 48 to 72 h, colonies were counted, and percent survival compared to the unirradiated control was determined.

(iii) X-ray irradiation. Cultures were prepared as described for UV irradiation. Plates were exposed to various doses of radiation by using a Machlett OEG-60 X-ray tube operated at 50 kV and 20 mA, delivering a dose of 1 Gy/s.

(iv) HU. HU treatment was as described above for determination of MMS sensitivity, with 0.2 M HU (Sigma) added to the medium. In addition, transformants containing the indicated plasmids were streaked out onto solid medium containing 10 mM HU, and colony formation was assessed in 48 to 72 h.

Doubling time. Ten milliliters of medium was inoculated with 10 to 100 μl of saturated culture, and cells were grown overnight until early log phase (approximately 10^6 cells/ml). Over the next 6 h, aliquots were removed, iced, sonicated, and counted on a Coulter (Miami, Fla.) Multisizer II Particle Counter. Linear regression was performed on the $\log_{10}$ of cell number versus time to calculate doubling time and r^2 value for each data set.

α-Factor arrest. Cultures were grown overnight in leucine-deficient pH 4.0 medium. After pelleting, cells were resuspended in medium with 2 μM α-factor and incubated until >95% of the cells were arrested as single buds by visual inspection under light microscopy. Cells were pelleted and resuspended in medium containing 20 μg of pronase E per ml. At indicated times, aliquots were removed, fixed in 70% ethanol for 1 h, and stored. Propidium iodide staining was performed (27), and samples were analyzed on a Becton Dickinson (Franklin Lakes, N.J.) FACScan.

Assay of β-galactosidase activity. Transcriptional induction by *CHES1* in wild-type and *rad9* and *dun1* mutant strains was assayed by using a reporter construct containing the *RNR2* (ribonucleotide reductase 2) promoter upstream of β-galactosidase (pSE788) (12). β-Galactosidase levels were measured quantitatively in a chromogenic assay using yeast extracts and *o*-nitrophenyl-β-D-galactoside (ONPG) as a substrate (44). The activity was then normalized to the amount of protein assayed in each lysate by Bio-Rad protein assay. β-Galactosidase activity was measured for wild-type (CRY1), *rad9* (Y438), and *dun1* (Y286) strains containing the pSE788 reporter and either ADANS or *CHES1* before and after exposure to 0.1% MMS for 4 h in liquid media.

Suppression of a *mec1Δ* null strain. A *mec1Δ* strain (Y669) containing the wild-type *MEC1* gene on a plasmid was transformed with ADANS, *CHES1*, or a longer cDNA containing the DNA binding domain [*CHES1(db)*]. Transformants were selected on leucine-deficient plates and then transferred to plates containing 5-fluoro-orotic acid (5-FOA; PCR Incorporated, Gainesville, Fla.) at 0.1% to select against the *URA3* plasmid containing the yeast *MEC1* gene. The loss of the *MEC1*-containing plasmid (in the colonies grown on 5-FOA) was verified by loss of a *MEC1*-specific PCR product by using two sets of primers designed in the 5′ (GCTTTTCCAGCTGCTTATATCGATC and GAACTAGCAGCACTAGAA AATGCCGA) and 3′ (TCTTTCCATGATTGCGCAAGAT and TGGGCTTA AGGAAGTTCGATACG) regions of the *MEC1* gene. *mec1Δ* clones containing *CHES1* were restreaked from a 5-FOA plate onto leucine-deficient medium and used for further assays.

Effect of DNA damage on the release of G_2-arrested cells. Logarithmically growing cultures in leucine-deficient medium were transferred to 50% rich media (YM-1) for 3 h. Ten milliliters of this culture was pelleted by centrifugation at 2,000 rpm for 3 min and resuspended in 5 ml of rich medium containing nocodazole (10 μg/ml; Sigma) in 1% dimethyl sulfoxide. After 2.5 to 3.0 h of incubation at 30°C, approximately 87 to 96% cells were arrested with a large bud and contained an undivided nucleus with DNA content in the neck of the large-budded cells, as monitored by staining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) as described previously (1). The cells were pelleted once again and plated in duplicate onto nocodazole (10 μg/ml)-containing agar plates. One plate was UV irradiated at a dose of 70 J/m^2, while the second plate served as a control. The cells were then immediately harvested from the plates and washed twice with medium to remove nocodazole and to recover cells into liquid medium. Samples were collected at 20-min intervals during recovery, briefly sonicated, fixed with 70% ethanol for 1 h, and resuspended in 10 mM Tris–1 mM EDTA buffer. Samples were counted for the fraction of large-budded cells as previously described (49) and for nuclear morphology after DAPI staining (1).

Inhibition of protein synthesis. Nocodazole-induced G_2-arrested cells (described above) were exposed to cycloheximide (10 μg/ml; Sigma) beginning 15 min prior and then continuously, during and after UV exposure as previously described (58). The inhibition of protein synthesis after cycloheximide treatment was determined by incorporation of ^{35}S-labeled methionine (ICN Radiochemicals, Costa Mesa, Calif.) into acid-precipitable materials (21, 34) and was found to be greater than 97% suppressed. Arrest after DNA damage was monitored by following the percentage of cells with a large-budded morphology with no visible septum between the bud and mother cell. In control experiments, we detected a decrease in large-budded cells after release from nocodazole in cells exposed to cycloheximide. Our results of monitor of cells in this fashion contrast with the inhibition of complete cytokinesis (as measured by cell number after treatment with glusalase) by cycloheximide exposure as reported by Burke and Church (6).

Nucleotide sequence accession number. The GenBank accession number for the *CHES1* cDNA sequence is U68723.

RESULTS

Isolation of a human cDNA which suppresses the *mec1-1* mutation. The genetic screen to obtain potential human checkpoint cDNAs was based on previous observations (59, 62) that a temperature-sensitive allele of DNA ligase (*cdc9-8*) had a decrease in maximum permissive temperature from 30 to 23°C when coupled with mutations in either the *RAD9* or *MEC1* checkpoint gene. Therefore, suppression of either the *mec1* or *rad9* mutation by a human cDNA in the double-mutant strain should allow growth at 30°C. A human cDNA library derived from the U118 glioblastoma cell line cloned in the 2μm yeast expression vector ADANS was used to transform the double-mutant strains. The vector from this library, ADANS, contains a constitutive *ADH* promoter upstream of the human cDNA. Baseline experiments with the ADANS vector demonstrated growth at 30°C of approximately one colony per 10,000 and 3,000 transformants for *rad9Δ cdc9-8* and *mec1-1 cdc9-8* strains, respectively.

Preliminary screening using this strategy resulted in the isolation of human *CDC34* (42), a cDNA which suppressed *mec1-1 cdc9-8* for growth at 30°C but did not suppress any other *mec1-1* phenotype. In total, screening of approximately 300,000 library *rad9Δ cdc9-8* and 400,000 *mec1-1 cdc9-8* transformant strains for growth at 30°C resulted in a total of 45 and 65 colonies, respectively. Only three of these transformants (transformants 6 [*CDC34*], 42, and 23) demonstrated plasmid-dependent growth at 30°C. Further analysis revealed a single clone, transformant 23 (*tx23*) of the *mec1-1 cdc9-8* strain, which conferred plasmid-dependent growth at 30°C and suppressed other checkpoint phenotypes (as described below). As demonstrated in Fig. 1A, *tx23* confers significant colony growth

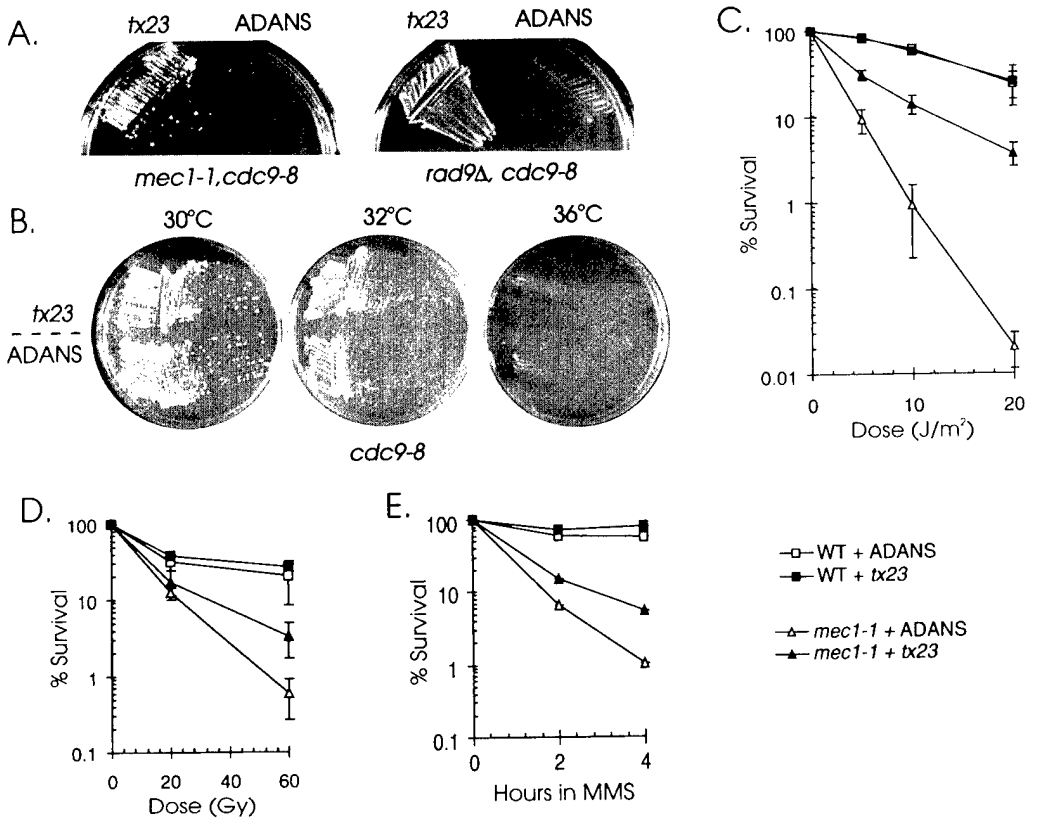

FIG. 1. Isolation of a human cDNA which suppresses multiple *mec1-1* phenotypes. (A) *tx23* rescues the temperature-sensitive lethality of *mec1-1 cdc9-8* (171-10-2) and *rad9Δ cdc9-8* (9085-1-8-3) double-mutant strains for growth at 30°C. (B) *tx23* does not rescue a *cdc9-8* (9085-1-10-4) strain for growth at 36°C. (C to E) Effects of UV light (C), X rays (D), and MMS (E) on the survival of wild-type (WT; 9085-1-7-3; □) and *mec1-1* (121-1-1b; △) yeast strains in the presence of an ADANS control vector (open symbols) or *tx23* cDNA (filled symbols). Graphical data shown in all figures are the averages and standard errors of means of two to four experiments. In some cases, e.g., panel E, the standard error of the mean is smaller than the icon size.

at 30°C for a *mec1 cdc9-8* strain compared to a library vector (ADANS) control. A similar suppression of the *rad9 cdc9-8* strain for growth at 30°C was also found (Fig. 1A). The cDNA did not directly suppress the *cdc9-8* mutation, as there was no change in the temperature-sensitive profile of a *MEC*$^+$ *RAD*$^+$ *cdc9-8* strain containing *tx23* (Fig. 1B).

Secondary assays for suppression of other DNA damage checkpoint phenotypes were then carried out. A common feature of strains containing mutations in DNA damage checkpoint genes is sensitivity to radiation-induced damage. In all screens of sensitivity to damage, exponentially growing fresh cultures in leucine-deficient medium were used. Transformation of a *mec1* strain with *tx23* resulted in 185-fold increase in survival after exposure to 20 J of UV radiation per m^2 (Fig. 1C). Similarly, survival after exposure to 60 Gy of ionizing radiation of both *mec1-1* (Fig. 1D) and *rad9Δ* (data not shown) strains was increased 10-fold by *tx23* compared with ADANS vector controls. There was no effect on survival of a wild-type strain containing *tx23* to either UV or ionizing radiation.

mec1 mutant strains have also been shown to exhibit defects in regulation of S phase after MMS and HU exposure. While treatment of *mec1* strains with MMS results in a S-phase arrest due to DNA damage (41), HU treatment is thought to trigger the S-phase checkpoint due to detection of stalled replication forks (60). Exposure of a *mec1-1* strain containing *tx23* to either HU or MMS revealed disparate results. *tx23* expression resulted in a fivefold increase in survival of *mec1-1* strains to MMS treatment (Fig. 1E). This difference was seen in multiple independent experiments. However, expression of *tx23* in this same strain had no measurable effect on HU sensitivity assayed both by survival of an exponential culture after 3 h of exposure to 0.2 M HU (0.17% for ADANS and 0.065% for *tx23* [Fig. 2A]) and by growth of wild-type and *mec1-1* mutant strains on plates containing 10 mM HU (Fig. 2B).

These experiments demonstrate that *tx23* is able to suppress multiple phenotypes in both the *mec1-1* and *rad9Δ* checkpoint-deficient strains. Given this activity, we named this cDNA *CHES1* (for checkpoint suppressor 1). To ascertain whether this effect is specific for the A364A strain background used in the *mec1-1* and *rad9Δ* strains described above, we tested the effect of *CHES1* on the same *rad9::HIS3* deletion allele in strain Y438, which is in a W303 strain background. *CHES1* expression in this background resulted in a 30-fold increase in survival of a *rad9* strain containing *CHES1* compared with an ADANS control when exposed to 20 J of UV radiation per m^2 (Fig. 3A). The result of these secondary screens demonstrate that *CHES1* suppression of *mec1-1* and *rad9Δ* strains is specific for the response of yeast to DNA damage and has significant activity in several strain backgrounds.

***CHES1* suppresses multiple checkpoint mutations.** Additional radiation-sensitive strains of *S. cerevisiae* were tested for suppression of UV sensitivity by *CHES1*. *RAD24* is in the same epistasis group as *RAD9*. However, Lydall and Weinert (36) presented data suggesting that *RAD24* plays a distinct role early in processing of DNA damage. Expression of *CHES1* also suppresses a *rad24-1* mutant strain when exposed to UV dam-

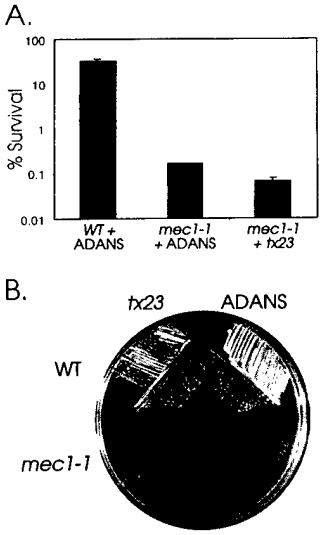

FIG. 2. *tx23* expression does not alter sensitivity of a *mec1-1* strain to HU. (A) Survival of wild-type (WT; 9085-1-7-3) and *mec1-1* (121-1-1b) strains containing ADANS control or *tx23* to 3 h of exposure to 0.2 M HU. (B) Survival upon exposure of transformants of wild-type and *mec1-1* strains to solid medium containing 10 mM HU.

age to a reproducible but lesser extent (fourfold) than it suppresses *rad9*Δ (Fig. 3B).

The protein encoded by *RAD53* (also isolated as *SPK1* [54], *SAD1* [4], and *MEC2* [60]) is an essential protein kinase which is required for both cell cycle arrest and transcriptional induction after DNA damage. Requirement for Rad53 activity has been placed downstream of Mec1 due to *RAD53* suppression of a *mec1* null allele and *MEC1*-dependent phosphorylation of Rad53 protein (46). There was a reproducible threefold suppression of the moderate UV sensitivity of a *rad53-21* strain (*sad1-1* allele) by expression of *CHES1* cDNA (Fig. 3C). *DUN1* encodes another protein kinase which is required for the transcriptional induction program after DNA damage (64). Sensitivity of *dun1*Δ strains to UV radiation is much greater than that of *rad53-21* strains, and this sensitivity was suppressed over 40-fold in multiple experiments by the presence of *CHES1* (Fig. 3D). A *dun1*Δ strain containing *CHES1* has resistance to UV radiation of 20 J/m^2 equivalent to that of an isogenic wild-type strain containing the ADANS control vector.

In contrast to the suppression of multiple checkpoint mutant strains, expression of *CHES1* did not suppress the sensitivity of strains mutant in two other radiation-sensitive epistasis groups required for DNA repair (43). There was no suppression of strains mutant in either *rad6* (Fig. 3E) or *rad52* (data not shown) for sensitivity to UV radiation.

The result of these secondary assays are summarized in Table 2. We have detected significant suppression of multiple aspects of the checkpoint response to DNA damage but not replication delays or repair defects by expression of *CHES1* in *S. cerevisiae*. This suppression results in increased resistance of the mutant yeast to DNA damage but does not change the response of wild-type yeast. The highest degree of suppression by *CHES1* is for *mec1-1*, *rad9*Δ, and *dun1*Δ strains exposed to UV radiation.

***CHES1* does not act by induction of DNA damage-responsive genes or constitutive effects on cell cycle kinetics.** We next explored the mechanism responsible for the increased survival of these mutant strains when *CHES1* is present. We considered three possibilities: (i) expression of *CHES1* results in constitutive induction of the DNA damage response genes, (ii) *CHES1* has a nonspecific effect on cell cycle kinetics, for example, slowing of the cycle, which makes the yeast strains more resistant; and (iii) *CHES1* results in reconstitution of the normal cell cycle arrest which accompanies DNA damage.

Given the finding that *CHES1* had significant suppression of a *dun1* mutant strain, we examined transcriptional induction after DNA damage. A reporter construct (pSE788) containing an *RNR2* promoter upstream of β-galactosidase was intro-

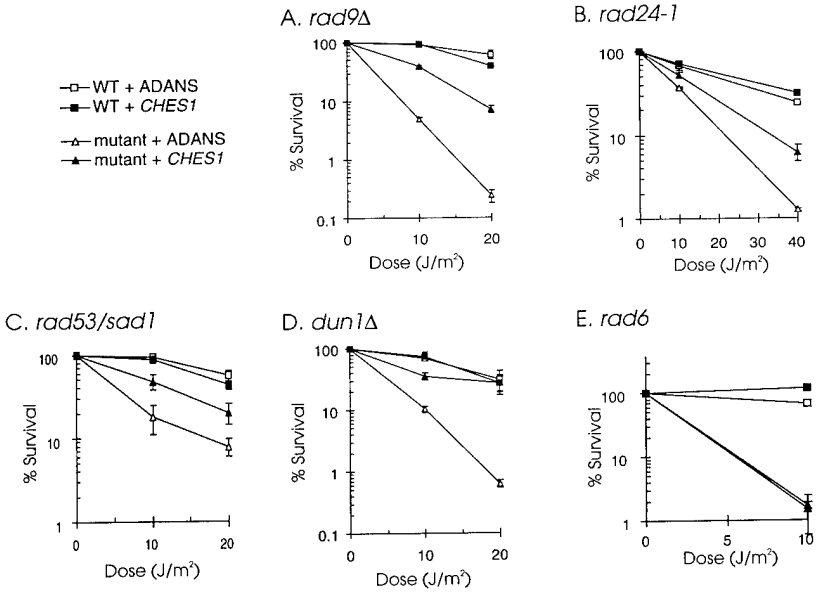

FIG. 3. *CHES1* suppresses the UV sensitivity of multiple-checkpoint-deficient but not repair-deficient strains. In all panels, the wild-type (WT) strain (CRY1 except for panel D, which is CRY2 to maintain the same mating type) is depicted by □; the mutant strains are indicated by △. (A) *rad9*Δ (Y438); (B) *rad24* (XRS255-1A); (C) *rad53-21* (Y301); (D) *dun1*Δ (Y286); (E) *rad6* (10011-2-1). Open symbols, ADANS control; closed symbols, *CHES1* cDNA.

TABLE 2. Summary of *CHES1* suppression of mutant phenotypes[a]

Relevant genotype	*cdc9-8* checkpoint	Ionizing radiation	UV	MMS	HU
mec1-1	+++	++	+++	+	−
rad9Δ	+++	++	+++		
dun1Δ			+++		
rad24-1			++		
rad53-21 (*sad1*)			+		
rad52Δ			−		
rad6			−		

[a] The symbols refer to the average degree of suppression of lethality compared with an ADANS control of the treatments indicated from analysis of between two and four independent experiments. −, no detectable suppression; +, 3- to 5-fold; ++, >5-fold; +++, >40-fold.

duced into these strains. This construct had previously been shown to be responsive to DNA damage in yeast (12), and this induction is lost in *dun1Δ* strains (64). Quantitative β-galactosidase activity was determined for wild-type (CRY1), *rad9Δ* (Y438), and *dun1Δ* (Y286) isogenic strains containing the reporter and either the ADANS or *CHES1* plasmid before and after exposure to MMS (Fig. 4A) and UV radiation (data not shown). There was no significant β-galactosidase induction of the exponential cultures prior to damage with either ADANS or *CHES1*. After damage, there was a small increase in induction of a wild-type strain carrying *CHES1* as opposed to ADANS but no increase in induction of either the *rad9Δ* or *dun1Δ* strain. Thus, rescue of the *rad9* and *dun1* mutations does not appear to be due to induction of this DNA damage-sensitive promoter either constitutively or after DNA damage.

To explore the effect of *CHES1* on cell cycle kinetics, we carried out quantitative assays to determine the doubling time of wild-type, *rad9Δ*, and *mec1-1* strains containing either ADANS or *CHES1*. In multiple assays, there were no significant differences between the doubling times of exponential cultures (Table 3). Second, we saw no differences in the profile of propidium iodide-stained cultures of exponential cells or cells after release from arrest in G_1 by α-factor (Fig. 4B and C). The *rad9Δ* cultures exited G_1 and progressed through S phase to G_2 with the same kinetics whether containing *CHES1* (Fig. 4C) or ADANS (Fig. 4B). Third, wild-type *mec1-1*, *rad9Δ*, and *dun1Δ* strains were synchronized in G_2 by nocodazole, and transit through the cycle upon release of G_2 synchronization was monitored by following the percentage of large-budded yeast with the nucleus in the neck after staining with DAPI. As shown in Fig. 5, undamaged cells of wild-type, *mec1-1*, *rad9Δ*, or *dun1Δ* strains containing either ADANS or *CHES1* when released from nocodazole block had superimposable exit from G_2. To summarize, on all three measures, exponential growth, release from G_1 arrest by α-factor, and release from G_2 arrest by nocodazole, there was no significant difference in cultures containing ADANS or *CHES1* compared with the large differences seen in survival after DNA damage in these strains.

***CHES1* restores the G_2 delay of *mec1-1* and *rad9Δ* strains damaged by UV irradiation.** Detailed assay of the damage-inducible checkpoint was studied by synchronization of cells in G_2 by addition of nocodazole (Fig. 5). These synchronized cells were then exposed to UV radiation, and the G_2 block was released by removal of nocodazole. Transit through the cycle was monitored by morphology and staining with DAPI. As expected (Fig. 5A), wild-type strains show significant delay after UV radiation with either ADANS or *CHES1*. In contrast, a *mec1-1* mutant strain (Fig. 5B) demonstrates complete loss of G_2 delay after UV radiation with the ADANS control vector.

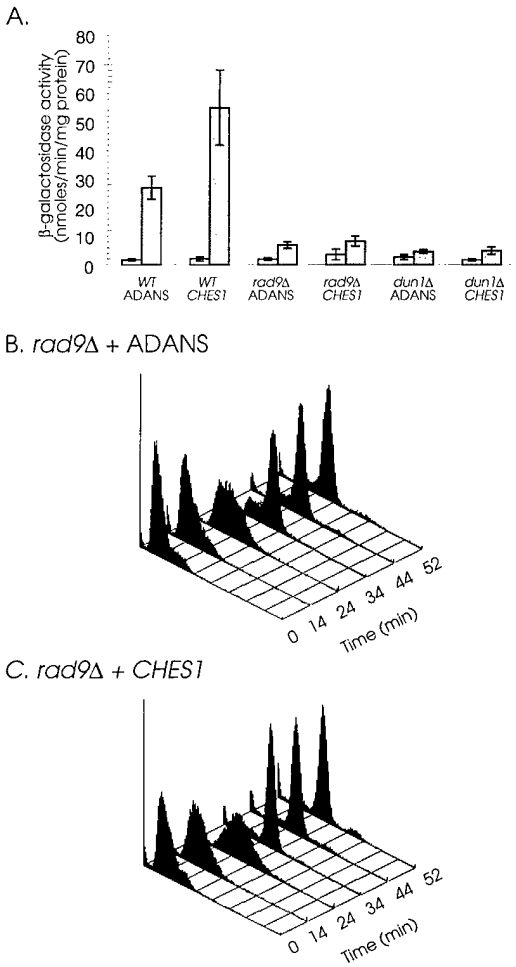

FIG. 4. Lack of transcriptional induction or cell cycle slowing by *CHES1*. (A) A reporter construct containing the *RNR2* promoter upstream of β-galactosidase was introduced in *rad9Δ* and *dun1Δ* strains. β-Galactosidase activity was measured for wild-type (WT; CRY1), *rad9Δ* (Y438), and *dun1Δ* (Y286) strains containing the reporter and either ADANS or *CHES1* cDNA after exposure to control medium (open bars) or medium containing 0.1% MMS (shaded bars) for 4 h. (B and C) An exponential culture of a *rad9Δ* (Y438) strain containing ADANS (B) or *CHES1* (C) was arrested with α-factor and then released into medium without α-factor. Samples were taken, fixed, stained with propidium iodide, and analyzed as described in the text.

Introduction of *CHES1* results in reconstitution of the G_2 delay, yielding a profile of G_2 arrest similar to that of the wild type. The same experiment was performed with wild-type, *rad9Δ*, and *dun1Δ* strains in a W303 background. *CHES1* also reconstituted the G_2 delay in *rad9Δ* strains to wild-type levels

TABLE 3. Doubling times of strains containing the *CHES1* cDNA or ADANS control

Strain	Plasmid	Doubling time (min)[a]
CRY1 (wild type)	ADANS	105
	CHES1	103
121-1-1b (*mec1-1*)	ADANS	126
	CHES1	128
Y438 (*rad9Δ*)	ADANS	101
	CHES1	109

[a] Based on experiments with $r^2 > 0.975$.

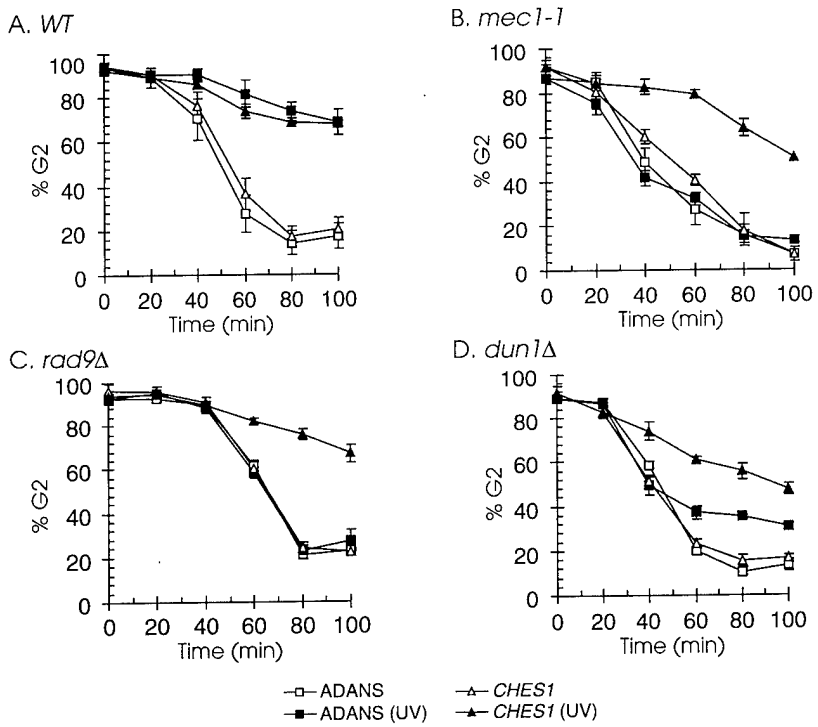

FIG. 5. Restoration of the DNA damage-induced G_2 checkpoint by *CHES1*. Nocodazole-arrested cultures were plated, UV irradiated, and then released into medium. Timed samples were fixed and analyzed as described in the text. Each panel represent analysis of one strain. (A) Wild-type (WT; CRY1); (B) *mec1-1* (121-1-1b); (C) *rad9*Δ (Y438); (D) *dun1*Δ (Y286). □, ADANS control; △, *CHES1* cDNA; filled symbols, UV-irradiated cells; open symbols, unirradiated controls.

(Fig. 5C) after UV irradiation. The *dun1*Δ strain containing the ADANS control had an intermediate loss of checkpoint function, with only 35% of the cells remaining in G_2 80 min after damage (Fig. 5D). Addition of *CHES1* resulted in an increase in G_2 delay in the *dun1*Δ strain to near wild-type levels. Thus, our analysis demonstrates no significant change in cell cycle control of undamaged cultures but substantial normalization of the G_2 checkpoint by *CHES1* upon damage of cells deficient in Mec1, Rad9, or Dun1 function.

***CHES1* encodes a truncated *fork head*/Winged Helix cDNA.** Analysis of the *CHES1* cDNA revealed a 1.6-kb message (Fig. 6A) with 600 bp of open reading frame placed in frame with the start site of translation and first 13 amino acids of the yeast *ADH* gene found in the ADANS vector. The remainder of the message was a 1.1-kb 3′ untranslated region (UTR) containing a CA dinucleotide repeat (see below). The cDNA was primed off of a short internal run of poly(A) in the 3′ UTR sequence. Analysis of the putative protein encoded by *CHES1* revealed a small but significant region of homology to HTLF (human T-cell leukemia enhancer factor), a member of the *fork head*/Winged Helix family (33). This possibility was further explored by obtaining longer cDNAs encoding an entire open reading frame (Fig. 6A) from human adrenal and T-lymphocyte cDNA libraries. This longer cDNA encodes a protein of 491 amino acids with predicted molecular mass of 54 kDa (Fig. 6B and C). Truncation of the cDNA to produce the original *tx23* clone occurred due to the presence of an internal *Not*I site in the cDNA.

The chromosomal location of *CHES1* was determined by use of the CA repeat in the 3′ UTR of the message. This repeat was found to be highly polymorphic in human DNA. Initial mapping determined linkage of this marker (named *CCC1*) with 14q32, which was further refined to the region between 14q24.3 and 14q31 (15). Flanking markers include D14S67 and AFM343.

Protein sequence analysis (5) with the open reading frame encoded by the longer *CHES1* cDNA revealed a high degree of homology to the DNA binding region of *fork head*/Winged Helix proteins. However, the DNA binding domain is not found in the original *CHES1* cDNA, *tx23* (Fig. 6B and C). An extensive analysis of 72 members of this family by both phylogenetic (14) and multiple alignment (53) methods centered on the shared DNA binding domain demonstrated that CHES1 belonged to a subfamily of *fork head*/Winged Helix proteins including HTLF, rodent WHN (40), and FKHR (18, 51). The alignment with HTLF, the closest relative of CHES1, is shown in Fig. 6C. Overall, there are 51% identical and 69% conserved residues between HTLF and CHES1. In addition to the highly conserved DNA binding domain, there are significant regions of homology between these two proteins both upstream and downstream of this motif, including portions encoded by the original truncated *tx23* cDNA.

Reconstitution of the G_2 checkpoint does not require new protein synthesis. Weinert and Hartwell previously demonstrated that the *RAD9*-dependent G_2 checkpoint did not require new protein synthesis (58). Although *CHES1* is a member of a family of transcription factors, the lack of the DNA binding domain in the truncated *CHES1* cDNA suggested that the *CHES1*-induced checkpoint would also be independent of new protein synthesis. UV irradiation of nocodazole-synchronized cultures was repeated with cycloheximide added before, during, and after irradiation. As shown in Fig. 7, *CHES1* reconstituted a G_2 delay in a *mec1-1* strain in the presence of cycloheximide with similar kinetics as in cells irradiated in the absence of cycloheximide.

A.

```
1                                30                                60
GAGGGTGGGTCGACCCCGGGAATTCGGCAC GAGCGGCGGCGGGGCCACCCGCGAGTCCAG CGTCGCCGCAGCCCCCCAATGCGGCCGCGA   90
GAAGCAGCGGGGGGGCAGGCGATCGAAGGA GCCTTCACGTAAATGGGTCCAGTCATGCCT CCCAGTAAGAAGCCAGAAAGCTCAGGAATT  180
AGTGTCTCCAGTGGACTGAGTCAGTGTTAC GGGGGCAGCGGTTTCTCCAAGGCCCTTCAG GAAGACGATGACCTCGACTTTTCTCTGCCT  270
GACATCCGATTAGAAGAGGGGGCCATGGAA GATGAAGAGCTGACCAACCTGAACTGGCTG CACGAGAGCAAGAACTTGCTGAAGAGCTTT  360
GGGGAGTCGGTCCTCAGGAGTGTCAGCCCC GTCCAGGACCTGGACGATGACACCCCCCCA TCCCCTGCCCACTCTGACATGCCCTACGAT  450
GCCAGGCAGAACCCCAACTGCAAACCCCCC TACTCCTTCAGCTGCCTCATATTTATGGCC ATCGAGGACTCTCCAACCAAGCGCCTGCCA  540
GTGAAGGATATCTACAACTGGATCTTGGAA CATTTTCCGTATTTTGCAAATGCACCTACT GGGTGGAAAAACTCAGTGAGACACAATTTA  630
TCATTGAATAAGTGTTTTAAGAAAGTGGAC AAAGAGAGGAGTCAGAGTATTGGGAAAGGG TCGTTGTGGTGCATAGACCCAGAGTATAGA  720
CAAAATCTAATTCAGGCTTTGAAAAAGACA CCTTATCACCCACACCCACACGTGTTCAAT ACACCTCCCACCTGTCCTCAGGCATATCAA  810
AGCACATCAGGTCCACCCATCTGGCCGGGC AGTACCTTCTTCAAGAGAAATGGAGCCCTT CTCCAAGATCCTGACATTGATGCTGCCAGT  900
GCCATGATGCTTTTGAATACTCCCCCTGAG ATACAAGCAGGTTTTCCTCCAGGAGTGATC CAAAATGGAGCGCGGGTCCTGAGCCGAGGG  990
CTGTTTCCTGGCGT tx23→
           GCGGCCGCTGCCAATC ACTCCCATTGGGGTGACAGCGGCCATGAGG AATGGCATCACCAGCTGCCGGATGCGGACT 1080
GAGAGTGAGCCATCTTGTGGCTCCCCAGTG GTCAGCGGAGACCCCAAGGAGGATCACAAC TACAGCAGTGCCAAGTCCTCCAACGCCCGG 1170
AGCACCTCGCCCACCAGCGACTCCATCTCC TCCTCCTCCTCCTCAGCCGACGACCACTAT GAGTTTGCCACCAAGGGGAGCCAGGAGGGC 1260
AGCGAGGGCAGCGAGGGGAGCTTCCGGAGC CACGAGAGCCCCAGCGACACGGAAGAGGAC GACAGGAAGCACAGCCAGAAGGAGCCCAAG 1350
GATTCTCTGGGGGACAGCGGGTACGCATCC CAGCACAAGAAGCGCCAGCACTTCGCCAAG GCCAGGAAGGTCCCCAGCGACACACTGCCC 1440
CTCAAAAAGAGACGCACCGAAAAGCCCCCC GAGAGCGATGATGAGGAGATGAAAGAAGCG GCAGGGTCCCTCCTGCACTTAGCAGGGATC 1530
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GTTTGGGGTTTGGTTTTCTTTCTTTTCTTT TCCTTTTATTGGGTTTTAATTTGCCCCCTC TTCTTTGTTTTGGACCCTTAAGAATTTTAT 1800
TTTTAAAGGAGATTGAAGCCATAGAACTCA TATTGACACTCAGCTGTTTTACAAAAGCTT TTCATTATCTGAAGACAAAACCGAAAAAGC 1890
CAAAATTACCATTGCTTCCTCCAGCTTGTC AGAAACCTGTGGCTGAATCCGCAGGGATGT CAACGTCAATATCACAGGAACACACATTCG 1980
GCACCTAGAAGGCACGTGGGCAAAGTAATC ATCGTTCAGGCCCAACCCTTAGGTTTAAAA AGTCAGGTTGTCCATCCCATTGGGGTTCAC 2070
TGAGTGAAGGCACATAAAGCAATTGAGGAG GAGGAGGAACCCCTCGTCCCCCTAGGAGCA GACCCAAGCTTGTGGCACCAGGCATCTGAT 2160
GGTGCCAGGAAAGCCACTGGAATTGTCACA CGGCGAGCACAGAGGGCCGGCCACCAGTCC TCGATGCTTCTGAACCCTGAACCCCGATGA 2250
CATCTTACGAGGTGGACGTTGGACTGTTCA TGCGCATCGGGTGTCAGTGACTCATGGAGA AGAAATGGGGTAAATTTTTAGTGATGTTGC 2340
TAATCATTGAATTCTGTTCTCTATTAAATT AAGAAAATGTTCCAAAAGCCATAAGCCTGA AGATTGGCCCTGTGCACGCACGCACACACA 2430
CACACACACACACACACACACACACACACA CACGAAGGAGAGAGAGAGAAAACTGATGGG GAAAACAAGCTGTGTCTTCTTAACTGCCCA 2520
AGTGAAAAGCAACCAAGTCCAGGAAATTAC AATAGCTGTTAAGGAAAGGAAATAATGGTA CAGATCTTTTTCTGTCTATCAAAACTATTT 2610
GATCCAAGTGAAAAAAAAAAAAAAACTAGA AAGCTACGGAACCTGCAATGCGGCCGC
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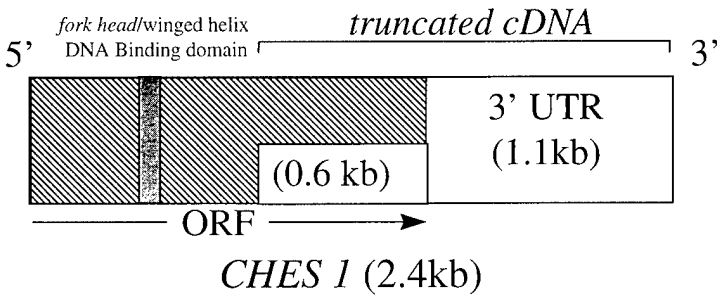
B.



C.

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      1                                                  46
CHES1 MGPVMPPSKKPESSGISVSSGLSQCYGGSGFSKALQEDDDLDFSL PDIRLEEGAMEDEELTNLNWLHESKNLLKSFGESVLRSVSPVQDL
                                                                                              *:::*
HTLF  ---------------------------------------------- -----------------------------------MMCHPLDQL

      91                                                 136
CHES1 DDDTPPSPAHSDMPYDARQNPNCKPPYSFSCLIFMAIEDSPTKRL PVKDIYNWILEHFPYFANAPTGWKNSVRHNLSLNKCFKKVDKERS
         *             ::    ******* **:**** ** * * ***:**:***:****** ********************:**:: ::
HTLF  ATRTQ----------KKKSATSKPPYSFSLLIYMAIEHSPNKCL PVKEIYSWILDHFPYFATAPTGWKNSVRHNLSLNKCFQKVERSHG

      181                                                226
CHES1 QSIGKGSLWCIDPEYRQNLIQALKKTPYHPHPHVFNTPPTCPQAY QSTSGPPIWPGSTFFKRNGALLQDPDIDAASAMMLLNTPPEIQAG
      :  *******:****: ******** *:                  *: * :*   :* : : :   *:::*****:*******:
HTLF  KVNGKGSLWCVDPEYKPNLIQALKKQPFS------SAS-----S- QNGSLSPHYLSSVIKQNQVRNLKESDIDAAAAMMLLNTS------

      271                tx23→                           316
CHES1 FPPGVIQNGARVLSRGLFPGVRPLPITPIG-VTAAMRNGITSCRM RTESEPSCGSPVVSGDPKEDHNYSSAKSSNARSTSPTSDSISSSS
                  : :*::   :***:       :  *::          *   :*   * *********::  :  * :*    *  **
HTLF  ------------IEQGILECEKPLPLKTALQKKRSYGNAFHHPSA VRLQE--SDSLATSIDPKEDHNYSASSMAAQRCAS---RSSVSSL

      361                                                406
CHES1 SSADDHYEFATKGSQEGSEGSEGSFRSHESPSDTEEDDRKHSQKE PKDSLGDSGYASQHKKRQHFAKARKVPSDTLPLKKRRTEKPPESD
      ** *: ***  * *: **:**** *:* *  :* : :*          *:*********          * :    : ** * :  :*:*
HTLF  SSVDEVYEFIPKNSHVGSDGSEG-FHSEED-TDVDYED------- --DPLGDSGYASQP--------CAKISEKGQSGKKMRKQTCQEID

      451
CHES1 DEEMKEAAGSLLHLAGIRSCLNNITNRTAKGQKEQKETTKN
       ** *************
HTLF  -EELKEAAGSLLHLAGIP-----------------------
```

FIG. 6. *CHES1* encodes a truncated *fork head*/Winged Helix transcription factor. (A) Sequence of the *CHES1* cDNA sequence. The ATG and TAA codons of the open reading frame (ORF) are highlighted. The original *CHES1* cDNA in transformant *tx23* began at one of two internal *Not*I sites in the gene. In addition, the polymorphic $(CA)_n$ repeat in the 3′ UTR is underlined. (B) Schematic diagram of the full-length *CHES1* cDNA. (C) Sequence alignment between *CHES1*- and *HTLF*-encoded proteins. Alignment was performed by using the ClustalW Alignment program (55). Amino acids in boldface represent the conserved DNA binding domain. *, identical amino acids, :, conservative changes. Conservative changes were defined as a relative value of >1 in the Dayhoff PAM matrix (19).

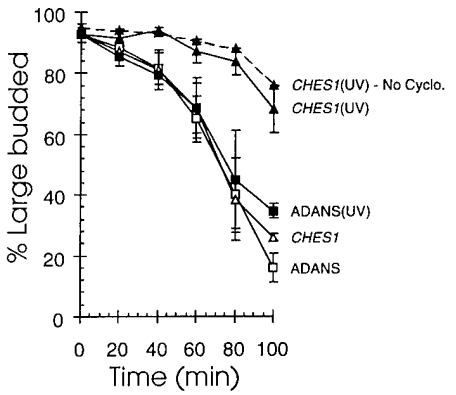

FIG. 7. Restoration of the *CHES1*-mediated G_2 delay of a *mec1-1* strain does not require protein synthesis. Nocodazole-arrested G_2 cells containing either ADANS (□) or *CHES1* cDNA (△) were exposed to the protein synthesis inhibitor cycloheximide (Cyclo.; 10 μg/ml) for 15 min before, during and after UV exposure (filled symbols). Open symbols, unirradiated controls. The broken line represents data from irradiated samples in the absence of cycloheximide.

***CHES1* suppresses the lethality of a *mec1* null mutation.** In addition to the multiple checkpoint phenotypes of a *mec1-1* strain, the *MEC1/ESR1* gene was also identified as being essential for growth in the absence of DNA damage (29). The essential requirement for *MEC1* has not been well characterized. We expressed both the *CHES1* and *CHES1(db)* cDNAs in a *mec1*Δ strain which contained the wild-type *MEC1* gene on a *URA3*-marked plasmid. Subsequently these transformants were plated on 5-FOA to select against the presence of the *URA3* plasmid. Expression of *CHES1* allowed numerous 5-FOA colonies to grow, in contrast to the lack of growth of colonies containing the ADANS control vector (Fig. 8A). Colonies containing the longer cDNA were visible about 1 day earlier than those containing the truncated *CHES1* cDNA. *CHES1*-containing colonies grown on 5-FOA were verified for the loss of the *MEC1*-bearing plasmid by using a robust PCR assay specific for the 5′ and 3′ ends of the *MEC1* gene. Of five *mec1*Δ *CHES1* colonies picked from the 5-FOA plate, we were unable to amplify either *MEC1* fragment (data not shown), confirming loss of the *MEC1* plasmid. The *mec1*Δ cells containing *CHES1* grow well with colonies first visible about 1 day later than the *mec1*Δ cells containing the *MEC1* gene. Thus, expression of *CHES1* is sufficient to overcome the essential requirement for *MEC1* function during growth. *mec1*Δ null mutants expressing *CHES1* demonstrated survival after UV irradiation (Fig. 8B) and a G_2 checkpoint response (Fig. 8C) similar to those of *mec1-1* cells expressing *CHES1*. This result clearly demonstrates that a checkpoint can be activated by *CHES1* in the complete absence of *MEC1* function.

DISCUSSION

Isolation of a human suppressor of multiple checkpoint mutations. In this paper, we report the isolation of a human cDNA, *CHES1*, which can substitute for several genes known to regulate the cell cycle response to DNA damage in yeast. The suppression of these mutant strains results in at least 10-fold increase in survival to several different forms of DNA damage. The greatest suppression (between 40- and 185-fold) is the UV sensitivity of *mec1-1*, *rad9*Δ, and *dun1*Δ strains. There is no suppression of UV sensitivity of the repair-deficient strains *rad6* and *rad52*. This suppression of the checkpoint strains is accompanied by reconstitution of a wild-type G_2 arrest after DNA damage in yeast strains deficient in this arrest due to mutations in the *MEC1*, *RAD9*, *RAD24*, *RAD53*, and *DUN1* checkpoint genes. Despite mutation in the known G_2 checkpoint pathway, the characteristics of this G_2 arrest are similar to those of the wild type in time course, nuclear morphology, and cycloheximide resistance.

mec1-1 strains remain very sensitive to HU upon expression of *CHES1*. HU sensitivity is reported to be due to loss of the checkpoint which senses incomplete replication (39). Although we were not able to detect suppression of HU sensitivity of a *mec1-1* strain, the truncated *CHES1* and longer *CHES1(db)* cDNAs are able to suppress the lethality of a *mec1*Δ strain. This result suggests that the essential requirement for *MEC1* may not be due the ability of Mec1 protein to sense replication delays.

In contrast to the ability of *CHES1* to reconstitute the G_2 checkpoint, expression of this clone does not restore the loss of transcriptional induction of an *RNR2* promoter after DNA damage in these mutants. We have found that in addition to a defect in transcriptional induction, the *dun1*Δ mutation results

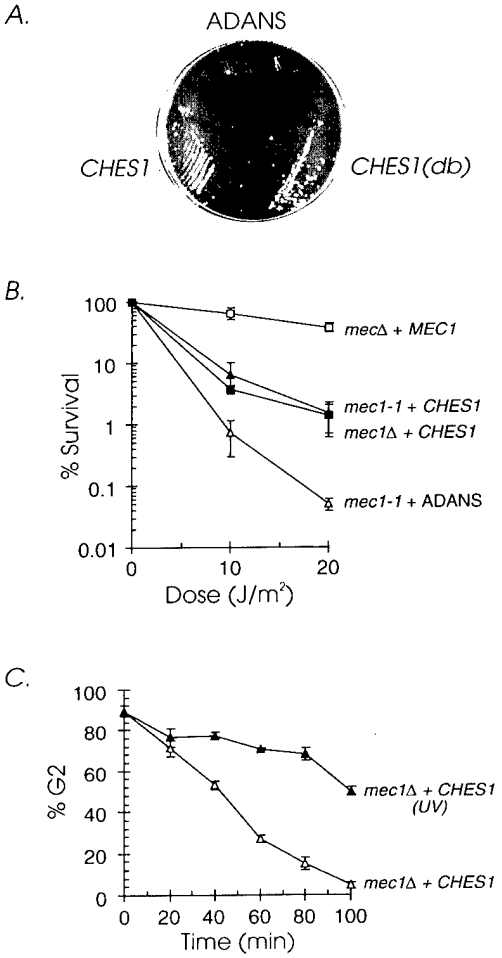

FIG. 8. *CHES1* suppresses a *mec1*Δ null mutant. (A) Expression of *CHES1* and *CHES1(db)* but not ADANS suppressed the lethality of a *mec1*Δ (Y669) strain, allowing growth of colonies on plates containing 0.1% 5-FOA, which selects against the *URA3*-marked plasmid containing the *S. cerevisiae MEC1* gene. (B) *CHES1* expression results in increased survival after UV irradiation. □, *mec1*Δ strain (Y669); △, *mec1-1* strain (121-1-1b); open symbols, ADANS; filled symbols, *CHES1*. (C) *CHES1* restores a G_2 checkpoint to a *mec1*Δ strain after UV irradiation.

in a partial G_2 checkpoint defect after UV irradiation. This checkpoint defect of a *dun1*Δ strain was not previously detected by Zhou and Elledge in assays using similar irradiation doses and strains (64). Restoration of this delay may explain the suppression of lethality in a *dun1*Δ strain by *CHES1* despite the lack of restoration of transcriptional induction.

***CHES1* encodes a novel *fork head*/Winged Helix protein.** Analysis of the *CHES1* cDNA reveals that this message encodes a novel member of the *fork head*/Winged Helix family of transcription factors and not a protein homologous to a known checkpoint gene. Similarly, Davey and Beach (10) identified a novel human cDNA, *RACH2*, in a screen for complementation of the *S. pombe rad1-1* mutation. We have no evidence that transcriptional induction of new genes is required for restoration of the checkpoint defect. First, only 200 amino acids in the carboxy terminus not including the conserved DNA binding motif are required for restoration of the checkpoint; second, the *RNR2* damage-inducible promoter is not activated by *CHES1*; and third, the G_2 checkpoint is restored in the presence of cycloheximide. Inclusion of a longer cDNA containing the DNA binding domain resulted in increased suppression of the checkpoint defect in *rad9* and *rad24* strains to wild-type levels (data not shown). However, this increased suppression may be a combination of the cell cycle slowing observed in cultures containing the longer *CHES1* cDNA (40a) and restoration of the DNA damage-induced G_2 checkpoint or the result of other factors such as increased stability of the protein.

The *fork head*/Winged Helix family was originally identified through studies of the *Drosophila* region-specific homeotic mutant *fork head* (57) and in studies of transcription factors which play a role in mammalian liver-specific gene (*HNF-3A*) expression (32). Members of this large family share a DNA binding domain which encodes a Winged Helix motif (57) but may have little homology outside this region. Although *S. cerevisiae* contains several genes with conserved *fork head*/Winged Helix motifs, there are no predicted proteins with significant homology to the carboxy terminus of CHES1. CHES1 also does not contain the *fork head*-associated domain first identified by short regions of homology outside the DNA binding domain in a subset of *fork head*-encoded proteins and many proteins involved in signal transduction, including Rad53 and Dun1 (26).

Sequence alignment and phylogenetic analysis of the *fork head*/Winged Helix family reveal that *CHES1* is most closely related to the mammalian genes *HTLF* (33), *FKHR* (18, 51), and murine *WHN* (40). *HTLF* shows significant homology to *CHES1* in the portion of the protein-coding region contained in the truncated *CHES1* cDNA. *HTLF* was first isolated in a screen for factors which bind to the long terminal repeat of the retrovirus human T-cell leukemia virus type 1. Further studies are needed to determine if *CHES1* or *HTLF* plays a role in the induction of viruses upon DNA damage in mammalian cells.

Evidence for an alternative *MEC1*-independent checkpoint pathway in budding yeast. The DNA damage-inducible checkpoint pathway in yeast can be divided into multiple steps including the sensor of damage and transduction of that signal, with subsequent cell cycle arrest and transcriptional induction (Fig. 9). Surprisingly, introduction of *CHES1* suppresses mutations which have been implicated in processing of damage (*rad9* and *rad24*) and in transduction of the signal (*mec1*, *rad53*, and *dun1*). The suppression of null alleles of *MEC1*, *RAD9*, and *DUN1* demonstrates that *CHES1* activity is not due to stabilization of the mutant gene products. Instead, our results suggest that in yeast *CHES1* must either act late in the pathway of response to damage after transcriptional induction or activate an alternative *MEC1*-independent pathway which results in G_2 arrest after damage. Examples of other alternative pathways in yeast include suppression of *rad6* UV sensitivity by overexpression of the *α1/a2* repressor (63). This suppression results from use of a *RAD52*-dependent recombination pathway. Similarly, there are rare survivors of *est1* mutations which escape senescence by maintaining telomere length through a parallel recombination pathway (35).

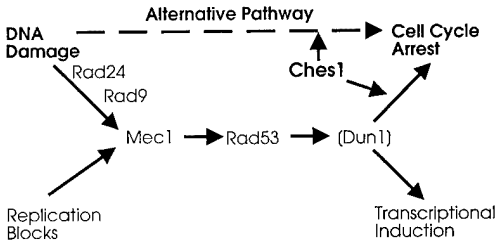

FIG. 9. A model for possible mechanisms of Ches1 action in cell cycle control and cellular response to DNA damage. Our data suggests that Dun1 may regulate both cell cycle arrest and transcriptional induction steps.

Our data support the alternate checkpoint pathway model due to the lack of detectable suppression of the *mec1* replication checkpoint (HU sensitivity) which would be expected for a protein acting downstream of *MEC1*. Activation of an alternative checkpoint pathway may not yield a phenotypic difference in wild-type strains due to an intact *MEC1*-dependent checkpoint pathway. Thus, expression of *CHES1* cDNAs in a wild-type strain would not be expected to yield a detectable difference in survival. The gene products required for the alternative checkpoint pathway can be further defined by identifying mutations which lose *CHES1*-dependent suppression of DNA damage checkpoints and proteins that interact with CHES1 in yeast after DNA damage occurs.

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Abstract - 2001

Eukaryotes possess well-conserved responses to DNA damage, including DNA damage-induced cell cycle arrests or "checkpoints." Our laboratory previously identified a human cDNA, *CHES1* (checkpoint suppressor 1), which encodes a forkhead/winged helix transcription factor that is sufficient to restore the DNA damage-induced G_2 arrest in multiple *S. cerevisiae* checkpoint mutants (Pati *et al*, 1997). Our data suggest that Ches1 functions independently of the primary *MEC1*-dependent checkpoint pathway. Our goals are to identify genes constituting this alternative pathway, to isolate the human counterparts, and to compare their structure and activity in normal and cancer tissues.

Only the c-terminal portion of *CHES1* lacking the DNA-binding domain is necessary for checkpoint activity. Full-length *CHES1* and yeast two-hybrid (Y2H) DNA-binding bait constructs fused to the c-terminus of *CHES1* are toxic when expressed in yeast. Expression of a functional c-terminal Ches1-GFP fusion protein indicates that Ches1 localizes to the nucleus; therefore, the DNA-binding activity exhibited by full-length Ches1 and the Y2H constructs appear to be the cause of toxicity when expressed in *S. cerevisiae*. Further experiments are underway to confirm this finding.

In order to identify the genes involved in the alternative checkpoint pathway, we are performing an insertional mutagenesis screen by mutagenizing a checkpoint mutant strain carrying *CHES1* on a high-copy expression vector. Mutagenized colonies exhibiting checkpoint phenotypes in the presence of *CHES1* have lost their *CHES1*-dependent advantage and are selected for further characterization. The screen has yielded several clones representing mutations in genes involved in such processes as DNA recombination and repair, gene silencing, microtubule stability, and protein phosphorylation status.

In efforts to identify Ches1-interacting proteins by biochemical methods, yeast protein extracts are being used for Glutathione-S-Transferase (GST) pulldown assays to identify proteins that interact with a functional GST-Ches1 fusion protein. The assays have revealed several Ches1-interacting proteins, including a major component of the primary histone deacetylase complex in yeast. We have also created a *CHES1* bait construct for screening cDNA libraries via the cytoplasmic Y2H approach. Screening of a human fetal brain library resulted in one positive clone, which functions as a transcriptional regulator through its association with histone deacetylase complexes in human cells.